Amniotic Fluid Natriuretic Peptide Levels in Fetuses With Congenital Heart Defects or Arrhythmias

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Background: We have previously demonstrated that umbilical cord plasma natriuretic peptide (NP) levels reflect the severity of heart failure (HF) in fetuses with congenital heart defects (CHD). The aim of this study was to evaluate the significance of amniotic fluid (AF) NP levels in the assessment of HF in fetuses with CHD or arrhythmia.

Methods and Results: This was a prospective observational study at a tertiary pediatric cardiac center. A total of 95 singletons with CHD or arrhythmia, and 96 controls from 2012 to 2015 were analyzed. AF concentrations of atrial NP (ANP), B-type NP (BNP) and N-terminal pro-B-type NP (NT-proBNP) at birth were compared with ultrasonographic assessment of fetal HF using the cardiovascular profile (CVP) score. Multivariate analysis showed that a CVP score ≤5 and preterm birth are independently associated with high AF NT-proBNP levels. AF NT-proBNP levels of fetuses with CHD or arrhythmia inversely correlated with CVP score (P for trend <0.01). In contrast, AF concentrations of ANP and BNP were extremely low, and it was difficult to assess the degree of fetal HF based on them.

Conclusions: AF NT-proBNP concentrations increase in stepwise fashion with the severity of HF in fetuses with CHD or arrhythmia; it was the optimal NP for assessing the fetal HF.

Key Words: Amniotic fluid; Arrhythmias; Congenital heart defects; Heart failure; Natriuretic peptides

Diagnosis of fetal heart failure (HF) is still challenging because it is difficult to know how well the fetal myocardium will perform its pump function under changing load conditions.1 Recently, the cardiovascular profile (CVP) score was found to be a good marker for a comprehensive semiquantitative assessment of fetal HF manifesting as fetal hydrops.3 The CVP score has been studied in relation to prognosis in fetuses with congenital heart defects (CHD).4,5 In pediatric and adult cardiology, atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and N-terminal pro-brain natriuretic peptide (NT-proBNP) are established markers of HF.7,8 Our previous study investigating the correlation between plasma NP levels and HF assessed by CVP score in fetuses with CHD or arrhythmia demonstrated that plasma NP levels reflect the severity of fetal HF.10 A previous report has suggested that urinary BNP levels are as useful as plasma BNP levels for diagnosing HF in adult patients.11 Because amniotic fluid (AF) is composed predominantly of fetal urine after 25 weeks of gestation, AF obtained after this time may provide useful information regarding fetal cardiac function.12 Several publications have reported NT-proBNP concentrations in AF samples in twin-twin transfusion syndrome (TTTS);13–16 all investigators found good correlation between AF NT-proBNP levels and Quintero stage or echocardiographic parameters of TTTS. Moriichi et al investigated AF BNP in monochorionic diamniotic (MD) twin pregnancies without TTTS at birth and found elevated AF BNP levels in patients with cardiac dysfunction monitored by postnatal echocardiography and in newborns with >20% weight difference.17 However, there is no published study investigating the correlation between AF NP levels and HF in fetuses with CHD or arrhythmia.
The aim of the present study was to evaluate the clinical significance of AF NP levels in the assessment of fetal HF and to identify the most informative diagnostic marker by comparing them with the pathophysiological status of fetuses with CHD or arrhythmia. We prospectively observed changes in CVP scores in utero and measured the AF NP levels at birth.

**Methods**

A single-center prospective observational study was undertaken with approval from the institutional review board (M24-041) and written informed consent was given by the parents. All singletons prenatally diagnosed with CHD or arrhythmia that was confirmed after birth at the National Cerebral and Cardiovascular Center of Japan between October 2012 and December 2015 were enrolled in this study. Exclusion criteria included a critical chromosomal anomaly such as trisomy 13 or 18, and a critical extracardiac anomaly that required surgical intervention during the neonatal period. Control subjects were randomly recruited from normal fetuses with no complications such as CHD, extracardiac anomaly or growth restriction. Maternal and obstetrical complications such as chronic hypertension, diabetes mellitus, preeclampsia, and gestational diabetes mellitus were also excluded from cases and controls. When the volume of AF samples was not enough for the measurement of NP because of technical problems with sampling, these samples were not tested for NP and excluded from the analysis (we defined these cases as ‘sampling failure’).

Our tertiary pediatric cardiac center has an established protocol for patients after a prenatal diagnosis of CHD or arrhythmia. All fetuses with CHD were diagnosed prenatally using fetal echocardiography performed with Voluson E8 ultrasound equipment (GE Medical Systems, Zipf, Austria). All fetal arrhythmias were categorized as tachyarrhythmia, bradyarrhythmia, or extrasystole. Fetal tachy- and bradyarrhythmias were defined by a ventricular rate ≥180 beats/min and <100 beats/min, respectively. When fetal tachyarrhythmia was sustained for ≥50% of the time on monitoring before 37 weeks of gestation, fetal treatment was performed. When complete atrioventricular block was complicated by a fetal ventricular rate <55 beats/min with or without myocarditis before 34 weeks of gestation, fetal treatment was performed. Details of fetal treatment were described in our previous study. Extrasystole was defined as a premature contraction independent of the normal rhythm and classified as atrial or ventricular by origin.

The CVP score was used as ultrasonographic assessment for fetal HF, as in previous studies. Briefly, the CVP score is based on a proposed composite scoring system to grade and serially follow the severity of fetal HF using 5 fetal echocardiographic parameters: fetal effusion, venous Doppler findings, heart size, cardiac function, and arterial Doppler findings. HF severity is rated on a 10-point scale; points are deducted for abnormalities in each component marker. A CVP score ≥8 is considered to indicate no or mild HF, 6 or 7 moderate HF, and ≤5 severe HF. CVP scores of all cases were evaluated by the same person. All fetuses were assessed with a CVP score within 1 week before birth.

Approximately 20 mL of AF were collected immediately after rupture of the membrane at the time of vaginal delivery or direct puncture at the time of cesarean delivery. Serial transabdominal sampling of AF before delivery was not done in this study for ethical reasons and the invasive nature of the technique. Approximately 20 mL of umbilical vein (UV) blood were collected immediately at the time of delivery into test tubes containing EDTA-2Na and aprotinin (final concentration: 1.5 mg/mL and 500 kallikrein inhibitor units/mL). After chilling AF and UV blood samples on ice, AF supernatant and plasma samples were prepared by centrifugation at 1,500 g for 15 min at 4°C and stored at −80°C until measurement. Each of 4–5 mL AF sample was further concentrated 20-fold using a Sep-Pak C18 cartridge (Waters, Milford, MA, USA). Both ANP and BNP concentrations in the AF and UV plasma were measured using the AIA-PACK chemiluminescence immunoassay (Tosoh Corp., Tokyo, Japan). An electrochemiluminescence immunoassay (Elecsys NT-proBNP II, Roche Diagnostics, Basel, Switzerland) was used to assess NT-proBNP concentrations in the AF and UV plasma.

**Statistical Analysis**

All analyses were performed using Stata 14.1 (StataCorp LP, College Station, TX, USA) and JMP 11 (SAS Institute, Cary, NC, USA) for statistical analysis. Continuous variables were compared using the Student t-test for normally distributed data, or the Mann-Whitney U test for non-normally distributed data. Categorical variables were compared using the chi-square test or Fisher’s exact test as appropriate. ANP and BNP concentrations were log-transformed to normalize their distribution. Multivariate binary logistic regression analyses were performed to assess the diagnostic value of AF NP levels for fetal HF after adjusting for maternal factors (e.g., age, weight, smoking status, etc.). The levels of statistical significance were determined at a p-value <0.05.
Cary, NC, USA). Data are presented as mean±standard deviation or numbers of patients. Student’s t-test was used to compare continuous variables between groups. Categorical variables were evaluated using the chi-square test or Fisher’s exact test as appropriate. Correlation analysis was performed using the trend test or Pearson’s coefficient. AF NT-proBNP concentrations in multiples of the median (MoM) were adjusted by gestational age in control fetuses. We also performed univariate and multivariate logistic regression of NP levels in fetuses with CHD or arrhythmia. The best prediction model was selected by stepwise analysis was used to adjust for baseline variables. P<0.05 was considered significant in all analyses.

Results

Study Cohort and Baseline Characteristics

A total of 143 fetuses with CHD or arrhythmia and 137 controls were prospectively enrolled in the present study (Figure 1). In the CHD and arrhythmias groups, 4 cases of fetal death, 3 cases of trisomy 18, and 41 cases with sampling failure were excluded, leaving 95 and 96 fetuses available for analysis. One fetal death was from Ebstein’s anomaly with a mean ± standard deviation of 32.0 ± 5.0 and 32.9 ± 4.5 years for the CHD and control groups, respectively (P<0.01). After adjusting the MoMs by gestational age at birth, and birth weight were shown as mean ± SD. Data are n (%) unless otherwise specified. AF, amniotic fluid; CHD, congenital heart defect; CVP score, cardiovascular profile score; SGA, small for gestational age; UA, umbilical artery.

Univariate and Multivariate Analyses of AF NP Levels

To identify perinatal factors associated with high AF NT-proBNP levels, univariate and multivariate analyses were performed for fetuses with CHD or arrhythmia (Table 3). Multivariate analysis showed that a CVP score ≤7 (coefficient 105.25, 95% confidence interval (CI) 24.22–186.27) and preterm birth (coefficient 73.31, 95% CI 22.72–123.91) were independently associated with high AF NT-proBNP levels.

AF NP Levels and Fetal HF

Fetuses with CHD or arrhythmia had 2.4-fold higher AF NT-proBNP levels than controls (97.2 ± 178.0 pg/mL vs. 40.9 ± 28.0 pg/mL, P=0.01). After dividing these fetuses into 3 groups by CVP score ≥7 (n=79), 6 ≤7 (n=11), and ≤5 (n=5), we found that the AF NT-proBNP levels of cases inversely correlated with CVP score (P for trend <0.01) (Figure 2A). No differences were observed in AF NT-proBNP levels between fetuses with a CVP score ≥7 and controls (P=0.22). After adjusting the MoMs by gestational age in the controls of this study, AF NT-proBNP levels of all cases inversely correlated with CVP score (P for trend <0.01) (Figure 2B). In fetuses with CHD or arrhythmia, a strong correlation between AF and UV NT-proBNP

Table 1. Perinatal Characteristics (n=191)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n=96)</th>
<th>CHD or arrhythmia (n=95)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, years</td>
<td>33.9±4.5</td>
<td>32.0±5.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Primipara status</td>
<td>34 (35.4)</td>
<td>49 (51.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>In vitro fertilization</td>
<td>9 (9.4)</td>
<td>10 (10.5)</td>
<td>0.81</td>
</tr>
<tr>
<td>Last CVP score</td>
<td>10.0±0</td>
<td>8.7±1.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Last biophysical profile score</td>
<td>10.0±0</td>
<td>9.4±1.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AF index, cm</td>
<td>12.9±3.6</td>
<td>13.3±4.7</td>
<td>0.59</td>
</tr>
<tr>
<td>Polyhydramnios</td>
<td>0</td>
<td>3 (3.2)</td>
<td>0.12</td>
</tr>
<tr>
<td>Oligohydramnios</td>
<td>0</td>
<td>3 (3.2)</td>
<td>0.12</td>
</tr>
<tr>
<td>Cesarean delivery</td>
<td>73 (76.0)</td>
<td>45 (47.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Labor</td>
<td>32 (33.3)</td>
<td>60 (63.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Gestational age at birth, weeks</td>
<td>37.9±1.1</td>
<td>38.0±1.4</td>
<td>0.46</td>
</tr>
<tr>
<td>Preterm birth</td>
<td>3 (3.1)</td>
<td>10 (10.5)</td>
<td>0.04</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>2,920±321</td>
<td>2,733±453</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SGA &lt;10th percentile</td>
<td>0</td>
<td>27 (28.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Male</td>
<td>53 (55.2)</td>
<td>50 (52.6)</td>
<td>0.72</td>
</tr>
<tr>
<td>Neonatal death within 1 month</td>
<td>0</td>
<td>1 (1.1)</td>
<td>0.31</td>
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<td>Infant death from 1 to 3 months</td>
<td>0</td>
<td>2 (2.1)</td>
<td>0.24</td>
</tr>
<tr>
<td>Apgar score ≤7 at 5 min</td>
<td>1 (1.0)</td>
<td>7 (7.4)</td>
<td>0.04</td>
</tr>
<tr>
<td>UA pH &lt;7.15</td>
<td>0</td>
<td>3 (3.2)</td>
<td>0.12</td>
</tr>
<tr>
<td>Ductal dependence</td>
<td>0</td>
<td>27 (28.4)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*P<0.05 indicates a significant difference. Maternal age, CVP score, biophysical profile score, gestational age at birth, and birth weight are shown as mean ± SD. Data are n (%) unless otherwise specified. AF, amniotic fluid; CHD, congenital heart defect; CVP score, cardiovascular profile score; SGA, small for gestational age; UA, umbilical artery.
levels was observed (r=0.81, P<0.01); NT-proBNP levels in AF were approximately 1/30 of the levels in UV blood (Figure 3A). In the controls, the correlation between AF and UV NT-proBNP levels was relatively weak but significant (r=0.51, P<0.01); NT-proBNP levels in AF were approximately 1/22 of levels in UV blood (Figure 3B). In controls, mode of delivery and labor were not associated with AF NT-proBNP levels, respectively (data not shown).

AF ANP and BNP levels were much lower than AF NT-proBNP levels. There was no difference in AF ANP and BNP levels between fetuses with CHD or arrhythmia and controls (AF ANP: 1.6±9.5 pg/mL vs. 0.8±1.7 pg/mL, P=0.49 and AF BNP: 5.3±8.3 pg/mL vs. 4.7±3.5 pg/mL, P=0.56). In the cases, neither AF ANP nor BNP levels correlated with CVP score (Figure S1A, B). In addition, no correlation was found between AF and UV ANP levels (Figure S2A) and between AF and UV BNP levels (Figure S2B). In the controls, mode of delivery and labor were not associated with AF ANP and BNP levels, respectively (data not shown).

**AF NP Levels and Changes in CVP Score In Utero**
A total of 13 fetuses with tachy- or bradyarrhythmia had an increase in CVP score in utero (from 6.8±1.6 to 9.3±1.4)
resulting from fetal treatment, while 14 fetuses with CHD or arrhythmia had a decrease in CVP score (from 7.0±2.5 to 6.0±2.3). In the CHD or arrhythmia group, fetuses with a decrease in CVP score had moderate or severe atrioventricular valve regurgitation (6/14, 42.9%) and tachy- or bradyarrhythmia at birth (6/14, 42.9%). They had 5.2-fold higher AF NT-proBNP levels than fetuses without a decreased score in utero (279.4±404.2 pg/mL vs. 53.7±63.9 pg/mL, P<0.01).

**Discussion**

Our study demonstrated a strong correlation between the
NT-proBNP levels in the AF and UV, and that AF NT-proBNP levels increased in a stepwise fashion with the severity of HF in fetuses with CHD or arrhythmia. In contrast, AF concentrations of ANP and BNP were extremely low, making them less useful markers for assessing fetal HF.

Fetal HF and preterm birth were independently correlated with high NT-proBNP levels in the AF. Previous studies reported good correlation between AF NT-proBNP levels and Quintero stage or echocardiographic parameters of TTTS in MD twin pregnancies. However, regarding MD twin pregnancies, BNP release was affected by factors such as fetal hypoxemia and renin transfer involving placental shunting from the donor twin to the recipient twin. In addition, it is unclear whether BNP production in the amniotic membrane is altered by polyhydramnios or oligohydramnios accompanying TTTS. Thus, it has not been established whether AF NP levels truly reflect fetal HF. In the present study, we focused on singletons with CHD or arrhythmia, and systematically compared AF NP levels with fetal HF severity assessed by the CVP score. As a result, AF NT-proBNP levels were demonstrated to correlate well with the severity of fetal HF in fetuses with CHD or arrhythmia, even after adjusting the MoMs by gestational age in the control fetuses. Our findings further highlight the feasibility of prenatal diagnosis of HF using AF NT-proBNP.

AF NT-proBNP levels strongly correlated with UV plasma NT-proBNP levels. We found that fetal tachy- or bradyarrhythmias and right heart defects with moderate or severe tricuspid valve regurgitation were associated with high AF NP levels, similar to the UV plasma NT-proBNP levels in our previous study. Fetal tachy- or bradyarrhythmias and right heart defects with moderate or severe tricuspid valve regurgitation were also associated with a decrease in CVP score in utero. Therefore, simultaneous assessment of CVP score and AF NT-proBNP may be considered in such cases. On the other hand, there are some problems with using echocardiography to evaluate cardiac function in fetuses with CHD or arrhythmia. It is not always easy to accurately evaluate the severity of tricuspid valve regurgitation. The gap in timing between atrial contraction and atrioventricular valve closure because of fetal arrhythmia can affect venous Doppler sonography findings. In the future, a further large study should be performed to clarify whether AF NT-proBNP levels can complement echocardiographic assessment in fetuses with CHD or arrhythmia.

The major source of NPs in the AF is controversial. Fetal urine and lung fluid are reported to be contributors to AF volume and NP concentrations. Amniotic cells are also known to produce NPs. Our study showed that AF NT-proBNP levels correlated with the severity of fetal HF. In addition, in both cases and controls, AF and plasma NT-proBNP concentrations were mutually correlated, even though the AF had much lower NT-proBNP concentrations than plasma, similar to the correlation reported between urinary and plasma NT-proBNP concentrations in adult patients with HF. This result suggested that most of the AF NT-proBNP is derived from the fetal heart through transfer from blood to urine. However, gestational age should be taken into consideration in the assessment of AF NT-proBNP values; our cohorts had a gestational age of more than 34 weeks at birth. In normal fetuses with lower gestation age, the amniotic membranes are reported to be the main source of AF NPs. The reference values for AF NT-proBNP in normal fetuses was shown to gradually decrease as pregnancy progresses and plateau after 34 weeks of gestation at median levels of less than 55 pg/mL. Therefore, AF NT-proBNP derived from the fetal heart can be used to assess fetal HF. In contrast, because ANP and BNP are degraded more quickly than NT-proBNP and are cleared by the NP clearance receptor, it was difficult to assess fetal HF using ANP and BNP in the AF when present in markedly low concentrations.

Study Strengths and Limitations

Strengths First, we investigated the similarities and differences of 3 NPs in the AF, and found that AF NT-proBNP was the superior marker for assessing the severity of HF in fetuses with CHD or arrhythmia. Although NT-proBNP is released from cardiomcyocytes in equimolar amounts with BNP, it is not metabolized via the NP clearance receptor, thus the half-life of NT-proBNP is much longer than that of BNP. Although the degree of glycosylation and the profiles of NT-proBNP contribute to preventing metabolism via the NP clearance receptor and neutral endopeptidase in the blood, the discrepancy between AF and plasma in the ANP and BNP concentrations cannot be explained by the present research data. In our results using the AIA immunoassay (Tosoh Corporation), not only BNP but also ANP showed remarkably low concentrations in AF. Because the AIA immunoassay recognizes the ring structure that is common to ANP and BNP but not NT-proBNP, glycosylation was not directly related to those results. On the other hand, the degree of glycosylation and NT-proBNP of NT-proBNP could affect the high stability and concentrations in AF. There is a possibility that glycosylation of AF NT-proBNP may be advanced in fetuses with HF, as in adults with HF. Furthermore, the glycosylated proBNP in AF may also be measured by electrochemiluminescence immunoassay for Roche NT-proBNP. Therefore, in a future study, we would like to elucidate the molecular basis of the merit of NT-proBNP measurement in AF by comparing the molecular weights using gel filtration chromatography of immunoreactive NT-proBNP and by examining the changes in molecular weight when AF is subjected to deglycosylation treatment for complete digestion of O-glycosylation. Second, our center is one of the largest tertiary pediatric cardiac institutions in Japan, and a variety of complex CHDs and arrhythmias was included in the study cohort. In addition, all fetuses with CHD or arrhythmia were diagnosed prenatally with high accuracy, and serial assessment using CVP score was available. Thus, even though there was a limited number of severe cases, we were able to unambiguously reveal the relationship between AF NP levels and fetal HF, avoiding biases such as extracardiac abnormality and MD twin pregnancy pathophysiology.

Limitations First, only an electrochemiluminescence immunoassay (Roche Diagnostics Elecsys NT-proBNP II) was commercially available to assess NT-proBNP concentrations. Thus our measurement data may include proBNP of the major BNP form in plasma, and may have interfered with the degree of glycosylation, indicating that the concentration cannot be directly compared with data measured by current BNP immunoassays. Second, especially in vaginally obtained AF samples, the effects of
maternal blood and vaginal secretion cannot be completely eliminated, even though mode of delivery and labor were not associated with AF NP concentrations in this study. Third, there were many cases of AF sampling failure. Because this study was exploratory research to evaluate the clinical significance of AF NP levels in the assessment of fetal HF and to identify the most informative diagnostic marker, we selected less invasive sampling methods from an ethical point of view, resulting in 29% collection failure of AF at delivery. Lastly, we have not yet clarified whether AF NT-proBNP levels are useful for assessing the severity of fetal HF before 34 weeks of gestation. Amniocentesis will reduce the noted sampling problems and provide real-time information earlier in gestation. Amniocentesis is a common obstetric method, using a hollow needle inserted into the uterus, to screen for chromosomal abnormalities in the fetus. Compared with umbilical cord blood sampling, amniocentesis has a lower rate of complications and is technically easier to perform. Therefore, we believe our results will help optimize the design of larger multicenter prospective studies using CVP scores, and AF collection by amniocentesis should be planned to establish the proper timing of delivery and improve the prognosis of fetuses with CHD or arrhythmia.

In conclusion, AF NT-proBNP concentrations increased stepwise with the severity of HF in fetuses with CHD or arrhythmia; it was the optimal NP for assessing fetal HF.

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Disclosure
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**Supplementary Files**

**Supplementary File 1**

**Figure S1.** AF ANP and BNP concentrations and CVP scores in fetuses with CHD or arrhythmia.

**Figure S2.** Correlation between AF and UV concentrations of ANP and BNP in fetuses with CHD or arrhythmia.

**Table S1.** Perinatal characteristics in cases of amniotic fluid sampling failure (n=81)

Please find supplementary file(s):