# Early Anti-Angiogenic Proteins Expression in Amniotic Fluid of Twin Fetuses

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Multiple pregnancies are thought to be associated with a high incidence of perinatal complications such as preterm labor, preeclampsia and low birth weight. But the true mechanisms of these obstetric complications are still uncertain. The components of amniotic fluid reflect the pathophysiology features of the fetus. Amniotic fluid soluble fms-like tyrosine kinase 1(sFLT1), soluble endoglin (sENG), and adiponectin reflect the oxidative stress and pro-inflammatory status and are associated with preeclampsia and fetal growth restriction. We prospectively collected amniotic fluids during amniocentesis from singleton and twin pregnancies. Samples were analyzed for levels of sFLT1, sENG, and adiponectin by enzyme-linked immunosorbent assay. The levels of sENG and sFLT1 were significantly increased in twin pregnancies. Adiponectin was not significantly different between the two groups. These findings would suggest that twin fetuses suffer from more oxidative stress and pro-inflammatory status from the early trimesters.

■ **Keywords:** twin, endoglin, soluble fms-like tyrosine kinase, amniotic fluid, adiponectin, preeclampsia

Multiple pregnancies have been reported as a risk factor of preeclampsia, small for gestational age or preterm delivery, which is 2 to 3 times higher than the reported incidence for women with singleton pregnancies (Blickstein & Keith, 2003; Krotz et al., 2002; Sibai et al., 2000). Twin pregnancies also have relative low birthweights compared to singleton pregnancies (Ananth et al., 1998; Cohen et al., 1997). The number of twin births has gradually increased since last decade (Blickstein & Keith, 2005; Sullivan & Newman, 2004), but the true mechanisms of these adverse perinatal outcomes in twin pregnancies are still unclear. During early embryogenesis, amniotic fluid is secreted from maternal plasma and then passed through the fetal membrane. Before 20 weeks of gestational age, the fetal skin keratinization has not completed and the amniotic fluid components are free to diffuse bidirectionally between amniotic fluid and the fetus across fetal skin (Cho et al., 2007). Thus, analysis of the amniotic fluid proteins between 16-20 weeks of gestational age would reveal the maternal placental environment and early pathophysiology conditions of the fetus (Cho et al., 2007).

The sFLT1, also known as soluble vascular endothelial growth factor receptor 1 (VEGFR-1), is a truncated form of the VEGFR-1 gene that is secreted by the human placenta (Clark et al., 1998). Recent studies demonstrated that amniotic fluid and circulating and placental sFLT1 expression have been shown to be essential in the pathogenesis of preeclampsia (Levine et al., 2004; Venkatesha et al., 2006; Wang et al., 2010). Endoglin (CD 105), is a co-receptor of transforming growth factor (TGF) –β1 and β3, which abundant in cell membranes of vascular endothelium and syncytiotrophoblasts (Cheifetz et al., 1992; St-Jacques et al., 1994). Soluble Endoglin is a placenta-derived soluble form of endoglin. It has been reported that this hypoxia-inducible protein is strongly associated with

the subsequent development of preeclampsia (Levine et al., 2006).

Leptin and adiponectin are both adipokine, mainly produced by adipocytes, which is also secreted by human amnion cells into amniotic fluid (Masuzaki et al., 1997). Excess leptin and adiponectin secretion in amniotic fluid or placenta was shown to be associated with preeclampsia (Mise et al. 1998; Wang et al., 2010). Increased AF leptin concentrations have been reported in twin pregnancies (Perni et al., 2005). However, there is no previous study that has investigated the correlation between amniotic fluid levels of adiponectin in twin pregnancies.

In our previous study, we demonstrated that amniotic fluid sENG, sFLT1, leptin and adiponectin, were presented before the preeclampsia onset (Wang et al., 2010). These up-regulated proteins may be associated with the inflammatory process in utero environment (Wang et al., 2010). Given the increased risk for preeclampsia in multiple pregnancies as compared with singleton pregnancies, we sought to determine these anti-angiogenic proteins and adipokines in amniotic fluid of twin pregnancies, to test the hypothesis that the increased fetal proinflammatory processes might be present early in the second trimester of twin pregnancies.

## Material and Methods

We prospectively stored the amniotic fluids of pregnant women who underwent genetic amniocentesis at Chang Gung Memorial Hospital, Lin-Kou Medical Center, after informed consent was obtained from all patients. The study was approved by the local ethical and research committees. The indications for genetic amniocentesis included advanced maternal age, maternal request, abnormal first or second-trimester serum screening, or family history of chromosome abnormalities.

The gestational age was examined by ultrasonography at the time of amniocentesis. The amniotic fluid volume during amniocentesis was evaluated carefully and oligohydramnios or polyhydramnios during amniocentesis were excluded. The chorionicity of twin pregnancies was identified by ultrasound during 10–14 weeks on the basis of the presence or absence of the lambda sign and confirmed after delivery by histopathological examination of the placenta and membrane (Chang et al., 2010). All the enrolled cases presented with normal genetic karyotype.

### Procedures of Specimen Collection

Before amniocentesis was performed, written informed consent was obtained from all participants. The procedure of amniocentesis has been described previously (Wang et al., 2011). In this study, genetic amniocenteses were performed between 16 and 18 gestational weeks at our prenatal diagnosis center. Using a transabdominal approach, about 20 mL of amniotic fluid was aspirated from each patient. After centrifugation to collect amnio-

cytes for culture and cytogenetic studies, we stored 4 mL of fresh AF at –80ºC freezer.

### Immunoassay Procedures

Enzyme-linked immunosorbent assays (ELISA) were performed according to the manufacturer's instructions as previously described (Wang et al., 2010). Samples were assayed in duplicate in a 96-well plate precoated with capture antibodies individually directed against soluble endoglin, using commercial ELISA kits (R&D systems, Minneapolis, MN). The AF concentrations of sENG and adiponectin were measured without prior dilution. The minimal detectable limits of the assays for sEndoglin and adiponectin were 0.007 ng/mL and 0.246 ng/mL, respectively. The intra-assay coefficients of variation (CVs) for sEndoglin and adiponectin ELISA were 2.8% and 3.2%, respectively, and the inter-assay CVs were 6.9%, and 7.9%, respectively ($n = 20$).

The sFLT1 concentrations in amniotic fluid were determined using a commercial ELISA kit (Quantikine Human sVEGF R1 Immunoassay; R&D Systems). The samples of AF were diluted to 1:100 in the calibrator diluent buffer of the kit before measurement. The minimal detection limit of the sFLT ELISA was 3.5 pg/mL, the intra-assay CV was 2.6%, and the inter-assay CVs was 9.8% ($n = 20$).

### Statistical Analysis

In this study, we used Chi-square tests to compare categorical variables, and Student $t$ test to compare the independent groups. Pearson's correlation was used to determine the correlation between the amniotic fluid proteins levels with twin fetuses, and fetal birth weight. A probability value of less than 0.05 was considered to be statistically significant. The calculations were carried out using SPSS version 12 for Windows (SPSS Inc., Chicago, IL).

## Results

### Clinical Characteristics

Sixty-five pregnant women were enrolled in this study (40 with healthy normal singleton pregnancies and 25 with twin pregnancies). The clinical characteristics of the study populations are summarized in Table 1. All enrolled cases had normal karyotypes, without fetal malformations, intrauterine growth restriction, or maternal disease such as diabetes or preeclampsia.

Between the women with twin pregnancies and singletons, there was no significant difference in maternal age, parity, BMI at amniocentesis and delivery, smoking habits, gestational ages at amniocentesis, and blood pressure levels during amniocentesis and delivery. Gestational ages of the pregnancies were longer in the singleton cases, because twin pregnancies usually delivery by scheduled Cesarean section (38.7 ± 1.1 vs. 36.4 ± 0.7; $P = .001$). The birthweight of babies was significantly lower in twin pregnancies due to the earlier delivery.

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**TABLE 1**

Characteristics of Enrolled Subjects With Singleton and Twin Pregnancies

| | Singletons (n = 40)[1] | Twins (n = 25)[1] | P value[2] |
|---|---|---|---|
| Age (years) | 35.2 ± 1.9 | 34.7 ± 1.6 | NS |
| Primiparous (%) | 50 (72%) | 16 (70%) | NS* |
| BMI at amniocentesis (kg/m$^2$) | 22.0 ± 1.6 | 21.6 ± 1.5 | NS |
| GA at AC (weeks) | 17.5 ± 0.7 | 17.4 ± 0.5 | NS |
| SBP at AC (mmHg) | 112.9 ± 8.8 | 114.6 ± 9.6 | NS |
| DBP at AC (mmHg) | 73.4 ± 5.2 | 75.1 ± 5.3 | NS |
| SBP at delivery (mmHG) | 122.8 ± 7.6 | 125.7 ± 6.0 | NS |
| DBP at delivery (mmHG) | 77.7 ± 5.1 | 79.3 ± 4.9 | NS |
| GA at delivery (weeks) | 38.7 ± 1.1 | 36.4 ± 0.8 | <0.001 |
| Birthweight (gm) | 3092 ± 402 | 2619 ± 241 | <0.001 |
| Smoking (%) | 2 (6.6%) | 1 (3.3%) | NS * |

Note: *Abbreviations* BMI, body mass index; AC, Amniocentesis; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age
[1] The values shown are median ± standard deviation
[2] *NS*: not significant, defined as *P* value ≥ .05; *P* values are given only for significant differences.
* Chi-square test

**TABLE 2**

sENG, sFLT1 and Adiponectin Concentrations in Twins and Singletons

| | Singletons (n = 40)[1] | Twins (n = 25)[1] | P value[2] |
|---|---|---|---|
| sENG (ng/ml) | 0.69 ± 0.04 | 0.92 ± 0.12 | 0.03 |
| sFLT1 (pg/ml) | 49063 ± 3274 | 63171 ± 5759 | 0.03 |
| Adiponectin (ng/ml) | 17.73 ± 1.20 | 16.47 ± 2.24 | NS |

Note: [1] Results are represented as mean ± SEM (Twin A+ Twin B / 2)
[2] *NS*: not significant, defined as *P* value ≥ .05; *P* values are given only for significant differences (independent Student *t* test).

**Amniotic Fluid sENG, sFLT1 and Adiponectin Levels**

The amniotic fluid sENG, sFLT1, and adiponectin in twin pregnancies were presented as the average concentration (Twin A + Twin B/ 2). The amniotic fluid levels of sENG in twin pregnancies (*n* = 25) were elevated (0.92 ± 0.12 ng/ml), compared with singleton pregnancies (*n* = 40) (0.69 ± 0.04 ng/ml; Figure 1). The sFLT1 was also significantly increased in amniotic fluid of normal twin pregnancies (63171 ± 5759 pg/ml), compared to normal singletons (49063 ± 3274 pg/ml) (Figure 2). However, adiponectin was not significantly different between the two groups. The data are summarized in Table 2.

**Amniotic Fluid sENG, sFLT1, and Adiponectin Correlation Between Both Twin Fetuses**

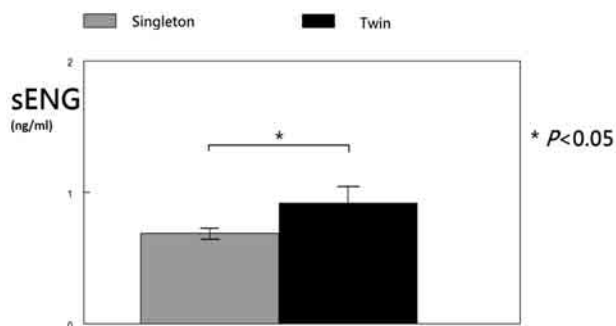We also performed the correlation of amniotic fluid sENG, sFLT1, adiponectin between the twin fetuses. In our study group, there was no significant correlation between the twin fetuses.

**Correlation Between Amniotic Fluid sFLT1, sENG, Adiponection and Fetal Birth Weight**

In this study, we also examine the relationship between amniotic fluid sENG, sFLT1, adiponectin and fetal birth weight. The amniotic fluid adiponectin levels were negative correlated with fetal birth weight(r = -0.389, P = .016). There were no significant correlation between the sENG, sFLT1 and fetal birthweight.

## Discussion

In this study, we found increased levels of sFLT1 and sENG in the amniotic fluid of healthy twin pregnancies, when compared to singleton pregnancies. These increased amniotic fluid anti-angiogenic proteins are thought to be associated with pro-inflammatory process (Wang et al., 2010). Some obstetrics complications, such as preterm labor, premature rupture of membrane and preeclampsia are all associated with inflammatory process. We propose that the relative elevation in pro-inflammatory proteins may contribute to the increased susceptibility of twin pregnancies to preeclampsia and preterm labor.

The sFLT1 is regulated by oxygen via hypoxia-inducible transcription factor 1, supporting a key role for low oxygen in regulating sFLT1 expression in the human placenta (Nevo et al., 2006). Reduced uterine perfusion in pregnant rats will increase sFLT1 production (Gilbert et
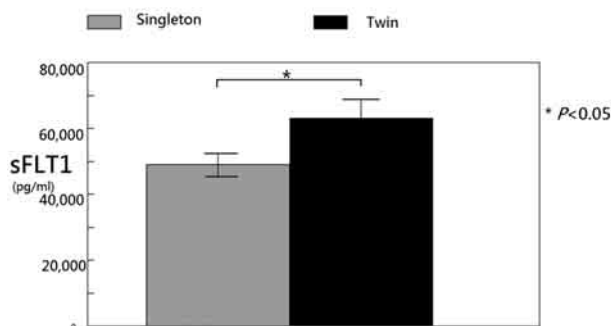


**FIGURE 1**

Amniotic fluid concentrations of sENG in singleton and twin pregnancies.



**FIGURE 2**

Amniotic fluid concentrations of sFLT1 in singleton and twin pregnancies.

al., 2007). These findings postulated that elevated sFLT1 reflect the condition of utero-placental hypo-perfusion and placental ischemic hypoxia. In our study, we detected the higher concentrations of sFLT1 in normal twin pregnancies mid-trimester amniotic fluid. We thought that the increased concentration of sFLT1 may reflect the relative hypoxic environment in twin pregnancies which may be associated the higher prevalence rate of preeclampsia in twin pregnancies.

Oxygen will also regulate the placental expression of endoglin via TGF-beta3. Reduced placental perfusion leading to placental hypoxia might contribute to the increased expression of endoglin (Yinon et al., 2008). In our study, we found that the increased sENG levels in normal twin amniotic fluids, which compared with normal singletons. This finding may demonstrate that the twin pregnancies suffer from relative low placental perfusion and oxygenation in twin pregnancies and explain the cause of relative low birth weight in twin pregnancy.

Leptin and adiponectin, which were both adipokine, are mainly associated with preeclampsia and IUGR (Wang et al., 2010). In twin pregnancies, increased amniotic fluid leptin was presented (Perni et al., 2005). In adiponectin, we could not find significant difference level of adiponectin between singleton and twin fetuses. In our previous study, we only found increased amniotic fluid adiponectin level in people with preeclampsia combined with IUGR group, but not without IUGR group (Wang et al., 2010). In addition, adiponectin was thought to be associated with fetal birthweight (Mazaki-Tovi et al., 2009). In this study, we also detected the adiponectin levels was negative correlated with fetal birthweight. We thought that the amniotic fluid adiponectin level was associated with fetal growth but not hypertensive disorder, such as preeclampsia. Therefore, we could not detect elevated adiponectin level in twin pregnancies.

In the correlation analysis, we could not find significant correlation of sENG, sFLT1 and adiponectin between the twin fetuses. However, we proposed that the sENG, sFLT1 would be regulated by oxidative stress, hypoxic change which resulted from unbalanced placental blood perfusion. Some twin fetuses would share the same placenta and would be suffered from unbalanced placental blood perfusion (Chang et al. 2011). Therefore, the unbalanced placental perfusion would result in different production of sENG and sFLT1.

In conclusion, we have found increased amniotic fluid levels of sENG and sFLT1 in twin pregnancies compared with normal singleton pregnancies. Our results suggest that early pregnancy in utero exposure to anti-angiogenic proteins is higher in twin pregnancies. The sFLT1 and sENG are all regulated by low oxygenation or placental hypoxia and poor placental perfusion and play an important role of pathophysiology in preeclampsia and reduced fetal growth. This finding would support the hypothesis that the twin fetuses suffer from poor placental perfusion and more hypoxic stress early in the second trimester. It may be responsible for the high incidence of obstetric complications in multiple pregnancies. We hope that the present work will provide evidence for and be used to elucidate underlying molecular mechanisms of the increased preeclampsia rate and low birth weight in twin pregnancies.

## Acknowledgments

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