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Increased free fetal DNA levels in early pregnancy plasma of women who subsequently develop preeclampsia and intrauterine growth restriction

S. Illanes[1,3]*, M. Parra[2], R. Serra[1], K. Pino[1], H. Figueroa-Diesel[1], C. Romero[2], J. A. Arraztoa[1], L. Michea[2] and P. W. Soothill[3]

[1]*Fetal Medicine Unit, Universidad de los Andes, Santiago, Chile*
[2]*Fetal Medicine Unit, Hospital José Joaquín Aguirre, Universidad de Chile, Santiago, Chile*
[3]*Fetal Medicine Unit, St Michael's Hospital, University of Bristol, Bristol, UK*

Objective To determine if maternal plasma ffDNA is increased early in pregnancies which subsequently develop preeclampsia (PE) and intrauterine growth restriction (IUGR).

Methods Blood was obtained at 11–14 weeks and plasma stored. Among those who delivered a male infant and had a birth weight under the tenth centile and/or PE, we divided them into those who delivered before 35 weeks (9) and those who delivered after this gestation (15). A third group with uncomplicated pregnancies was used as controls (24). Real time-polymerase chain reaction (RT-PCR) was carried out to detect the multi-copy Y chromosome associated DSY14 gene.

Results There were no differences between the ffDNA levels in the group delivered after 35 weeks and the control group (2.23ge/mL–1.61ge/mL $p = 0.39$). However, the levels of ffDNA at 11–14 weeks were statistically, significantly higher in patients that delivered before 35 weeks (4.34ge/mL–1.61ge/mL $p = 0.0018$). A logistic regression analysis shows that for every unit (1ge/mL) in which ffDNA increases, the likelihood of having PE or a fetus growing under the tenth centile delivered before 35 weeks increases by 1.67 times (CI 1.13–2.47).

Conclusion The concentration of ffDNA is significantly higher even during early pregnancy, in patients who subsequently develop PE and/or IUGR and are delivered before 35 weeks. Copyright © 2009 John Wiley & Sons, Ltd.

KEY WORDS: preeclampsia; maternal disease; placental disease; DNA; fetal Cells; nucleic acids and proteins; maternal serum Screening; fetal and placental pathology

INTRODUCTION

Preeclampsia (PE) and intrauterine growth restriction (IUGR) are prevalent disorders in pregnancy with a large impact in fetal and maternal morbidity and mortality. Despite advances in the understanding of the pathogenesis of these diseases, their pathophysiology has not yet been clearly elucidated. In PE, the disease constitutes a spectrum that includes so called 'maternal' and 'placental' PE (Ness and Roberts, 1996). In the maternal PE there is a systemic inflammatory response that involves all the components of the inflammatory circulation, including the endothelium (Borzychowski *et al*., 2006), but with a normal placentation. With placental PE, there is an abnormal placenta in a normal woman, whose etiology is poor trophoblastic perfusion generating oxidative stress and release of factors into the maternal circulation from the placenta due to trophoblast cell death. This process triggers an inflammatory response and endothelial dysfunction, which could produce the clinical syndrome of PE, or IUGR or both (Borzychowski *et al*., 2006). Numerous methods have attempted to predict PE by different biochemical markers of the inflammatory response and endothelial dysfunction; however, the best sensitivity and specificity are obtained when these tests are undertaken at the end of the second trimester of pregnancy, which restricts possible interventions to prevent the occurrence of the clinical disease (Fayyad and Harrington, 2005).

Thus, there is a need for markers that respond to the pathophysiology involved in the genesis of these conditions in order to predict those patients who will develop PE and IUGR in the early stages of the pregnancy. This has led to the exploration of using ffDNA as an early marker of placental dysfunction in patients with PE and IUGR. Data from clinical and *in vitro* studies support this view where elevated concentrations of ffDNA are found in conditions associated with significant placental pathology (Hahn *et al*., 2005), and the association between degenerative changes in the syncytiotrophoblast and the release of ffDNA has been demonstrated (Tjoa *et al*., 2006). The aim of this study is to determine if ffDNA is increased even in early pregnancy in the plasma of women who subsequently develop severe placental disease.

*Correspondence to: S. Illanes, Fetal Medicine Unit, Universidad de los Andes, Santiago, Chile. E-mail: s.illanes@bristol.ac.uk

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METHODS

Study design

From April 2002 to March 2005 at the Jose Joaquin Aguirre Hospital in Santiago, women attending for routine scanning at 11 and 14 weeks consented to provide a 20 mL blood sample for research purpose. Blood was transferred to the laboratory within 8 h at room temperature and plasma was separated and stored. All patients signed informed consent including the use of clinical data for research purposes and the protocol were approved by the Institutional Review Board of the University of Chile. The pregnancies were followed prospectively to determine the outcome and all clinical relevant data as the study was stored in a computer database. The patients with singleton pregnancies, which delivered a male infant and with a clinical diagnosis of PE or a birth weight under the tenth centile for Chilean normal growth reference curve (Juez *et al*., 1989), were used as cases. PE was defined as *de novo* hypertension and proteinuria after the 20th week of gestation. Hypertension was defined as a DBP $\geq$110 mmHg or two consecutive readings of $\geq$140/90 mmHg measured 6 h apart. Proteinuria was defined as a 24-h urine protein of $>$300 mg/day (Brown *et al*., 2001). The cases were subsequently divided into those who delivered before 35 weeks and those who delivered after this gestational age. A third group with normal pregnancy outcome, defined as a pregnancy that progressed without the development of any serious obstetric disease and delivery of a healthy male infant after 37 completed weeks, was used as control. The number of controls was decided to match the number of cases. There were no aneuploid fetuses in either groups.

Polymerase chain reaction testing

In the laboratory, maternal blood was centrifuged at 3,000 g for 10 min. The supernatant plasma layer was transferred to a fresh tube without disturbing the buffy coat and 900 μL aliquots of plasma were centrifuged for a further 10 min at 7,000 g. eight hundred microliters of plasma aliquots were transferred to another fresh tube without disturbing the cell pellet and were stored at −20 °C until use. DNA was extracted from the 800 μL plasma aliquots using the QIAmp Blood DNA Mini Kit (Qiagen, West Sussex, UK) and was eluted in 50 μL water. Real-time polymerase chain reaction (PCR) was performed to detect the multi-copy Y chromosome associated *DYS14* gene, as previously described (Lo *et al*., 1998). This target was used because the sensitivity for detecting low levels of DNA may be higher than when using a single copy PCR target such as SRY (Lambert *et al*., 2002). Three replicates of the *DYS14* reaction (DYS14 5′ sense CAT CCA GAG CGT CCC TGG 3′; DYS14-5′ antisense GCC CAT CGG TCA CTT ACA CTT 3′) were performed for each plasma sample. Five microliters of DNA was used for each replicate. The 25 μL PCR mix consisted of water (free of nuclease), PCR template, 3.5 mM $MgCl_2$, 3 mM dNTPs, 2U Taq (kit Go Taq Flexi, Promega), 2.5 μM DYS14-sense primers, 2.5 μM DYS14-antisense primers; SYBR Green 1/20000 (Molecular Probes). The PCR reaction was carried out by a thermocycler in a CHROMO 4 (MJ Research) using the following reaction conditions: 96 °C for 2 min, then 45 cycles at 92 °C for 20 s, and 54 °C for 20 s and extensions to 72 °C for 20 s. The data analysis was performed using OPTICON 111 software. Amplification of the target sequence, if present, was detected by a proportionate increase in reporter dye fluorescence, which is monitored throughout the PCR reaction. The cycle threshold (Ct) is the cycle number at which a detectable level of fluorescence is achieved (i.e. the point at which a specific number of copies of the target sequence have been produced) and so gives an indication of the amount of target DNA present with lower Ct indicating more DNA. Abundance of target DNA was calculated from standard curves obtained by serial dilution of known concentrations of PCR products used as template (correlation coefficient $\geq$0.98). To confirm amplification specificity the PCR product agarose gel electrophoresis and melting curve analysis were used.

Statistical analysis

ffDNA levels were expressed as genome equivalents per mL (GE/mL) of plasma. DYS 14 levels were compared between groups using one way analysis of Scheffe's test to compare among groups. To determine the association between ffDNA and the possibility of having a delivery of less than 35 weeks with PE and/or fetus growing under the tenth centile, we used a univariate logistic regression model. The dependent variable was dichotomized into patients with delivery before 35 weeks with these pathologies (PE/IUGR) and patients without this condition. Of the variables studied, we only included in the model fetal DNA level, since it is the only one that eventually would be predictive.

RESULTS

The patient characteristics of each group are presented in Table 1. The gestational age at delivery was similar in the control group and those affected and born after 35 weeks, but as expected the average birth weights were statistically different in the three groups, with the one born before 35 weeks the lowest.

In the pregnancies that delivered after 35 weeks, the ffDNA level was not significantly different from the control group (2.23 E/mL and 1.61 GE/mL $p = 0.39$). However, ffDNA levels at 11–14 weeks were statistically, significantly higher in patients that delivered before 35 weeks with PE or a birth weight under the tenth centile than both the controls and those with pathology delivered after 35 weeks (4.34 GE/mL v/s 1.61 GE/mL and 2.23 GE/mL $p = 0.0018$) (Figure 1).

The minimum and maximum values of fetal DNA used to estimate the logistic model were from 0.1 to 9.4.

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Table 1—Clinical characteristics of patient groups

Characteristic	Control (24)	PE/IUGR >35 weeks (15)	PE/IUGR <35 weeks (9)
Maternal age (years)	29	30.9	31.8
Gestation at delivery (weeks)	38 + 3	38 + 1	32 + 6*
Birth weight (grams)	3285	2659*	1672*

Data are given as average and compared using anova test.
* Differs significantly from control group ($p < 0.0001$).

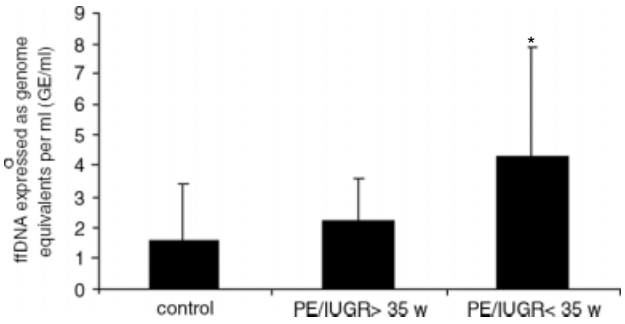

Figure 1—ffDNA levels at 11–14 weeks expressed as genome equivalents per mL (GE/mL) of plasma. The groups are controls and cases with preeclampsia (PE) and or a birth weight < tenth centile divided into those delivered at or after 35 weeks or before 35 weeks

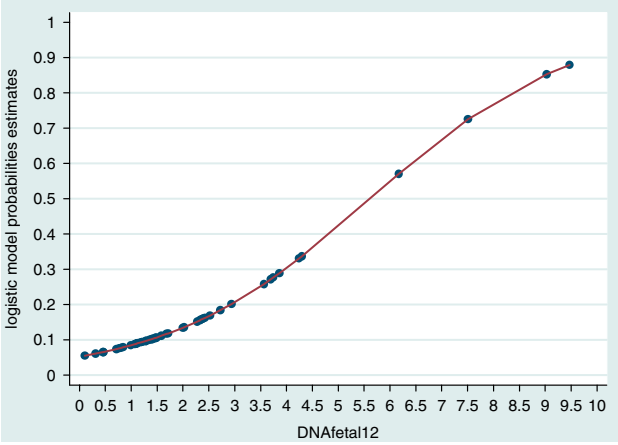

Figure 2—logistic regression probabilities estimates of PE/IUGR <35 weeks versus ffDNA (1GE/mL)

Logistic regression analysis showed that for every unit (1 GE/mL) of increase in ffDNA the odds ratio of having a patient with PE or a birth weight under the tenth centile and delivering at less than 35 weeks increases by 67% over the null value. This association was statistically significant ($p = 0.0027$) (1.67 CI 1.13–2.47) (Figure 2). This model has the ability of discrimination of 68%. The Hosmer-Lemeshow test showed that the model fits the data ($p = 0.2$).

DISCUSSION

We have shown that ffDNA is significantly increased between 11 and 14 weeks in patients who subsequently develop PE and IUGR and are delivered before 35 weeks, suggesting that ffDNA may be a marker of placental damage at an early stage of the disease.

There is a lot of evidence which indicate that the placenta is the main source of free fetal DNA in maternal plasma (Wataganara and Bianchi, 2004), and perhaps with a modest contribution from apoptosis of fetal hematopoietic cells. DNA is present in maternal plasma of normal pregnancies and increases with advancing gestation as a result of the physiological remodeling that occurs normally in the trophoblast (Tjoa *et al*., 2006). Studies *in vitro* demonstrate a clear association between degenerative changes mediated by apoptosis and necrosis occurring in the syncytiotrophoblast and release of this fetal DNA (Tjoa *et al*., 2006). On the other hand, trophoblast cell death is increased in both PE and IUGR (Huppertz *et al*., 2006) and a significant part of this occurs not only by apoptosis (Smith *et al*., 1997; Allaire *et al*., 2000; Erel *et al*., 2001; Ishihara *et al*., 2002), but also by necrosis (Formigli *et al*., 2000; Huppertz *et al*., 2006). There is an increase in villous exchange in patients with PE, with a raise in the proliferation and remodeling of the trophoblast. The consequence of these processes is a steady increase in the flow of placental material to the maternal circulation (Arnholdt *et al*., 1991) and probably a direct correlation between the degenerative changes in the syncytiotrophoblast and the levels of ffDNA (Tjoa *et al*., 2006). It is not well known when the changes in the placenta of patient who will develop severe PE primary occur; however, our results suggest it may be as early as in the first trimester.

Our results are in line with other publications that show an increase in concentration of ffDNA in the plasma of patients with PE before the start of the clinical symptoms (Lo *et al*., 1999; Leung *et al*., 2001; Zhong *et al*., 2001; Cotter *et al*., 2004; Levine *et al*., 2004; Alberry *et al*., 2009), however to our knowledge this is the first time that this has been described so early in pregnancy (11–14 weeks). Cotter *et al*. (2004) described this increase at a mean gestation of 16 weeks and Levine *et al*. (2004) showed this raise after 17 weeks. The gestational age at delivery in our patients is a marker of the severity of the clinical syndrome, showing the need of medical intervention because of fetal or maternal compromise. The fact that our results show only increased levels of ffDNA in the cases delivered before 35, suggest that ffDNA will increase only in the most severe cases. Alberry *et al*. (2009) have recently shown that ffDNA levels were significantly higher in patients with clinical diagnosis of PE and fetal growth restriction groups compared with normal pregnancies, but most importantly they were higher in cases of severe PE compared with those with milder disease. Levine *et al*. (2004) also showed differences in the amount of ffDNA but before the onset of the clinical disease, and these differences were greater if the PE was severe, or at an earlier gestational age or associated with a

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small gestational age infant. Moreover, these differences could be highlighting a different physiopathology for a similar clinical syndrome. There is a spectrum of disease that includes 'maternal' and 'placental' PE (Ness and Roberts, 1996). In general, in placental PE there is an abnormal placenta in a normal woman, usually with a more severe clinical syndrome. In maternal ones there is a normal placentation in a woman with a pre-existing condition, such as cardiovascular disease, chronic hypertension or diabetes, and frequently with a late and mild clinical onset of the PE. The placental type, whose etiology is a poor trophoblastic perfusion, which generates oxidative stress and release of placental factors to the maternal circulation (Borzychowski *et al*., 2006), is usually associated with more severe disease, and should be associated with increased levels of ffDNA in maternal plasma. On the other hand, the etiology of the maternal PE is a systemic inflammatory response that involves all the components of the inflammatory circulation, including the endothelium (Borzychowski *et al*., 2006), but without release of placental factors because there is a normal placentation, and so perhaps normal levels of ffDNA.

Before screening in pregnancy can be seriously considered the performance of the test and the possible benefits and risks must be established. However, despite detecting raised blood pressure and proteinuria, PE is still associated with serious morbidity and mortality. Accurate risk prediction might allow more focused monitoring, and also offer reassurance to lower risk women. Early management may avoid serious problems like abruption, renal failure or HELLP syndrome. In addition the controversy about the possible value of low dose aspirin or vitamin (Poston *et al*., 2006; Duley *et al*., 2007) to prevent or reduce this disease makes it clear that identifying the right cases to intervene may be crucial. The debate also highlights that any intervention will probably be needed from early pregnancy. Therefore the changes reported in this study offer the hope of progress in early identification of the worst prognosis (early delivery) cases.

A major problem for the use of ffDNA in screening is the absence of a marker of DNA quantity in female pregnancies (Lo *et al*., 1999; Alberry *et al*., 2009). However, recent studies (Chim *et al*., 2005) have shown that the determination of epigenetic changes, such as differential gene methylation in the placental tissue versus the maternal tissue, could be used as a universal method for detecting fetal DNA in maternal plasma.

In summary the levels of ffDNA seem to be elevated early in cases which subsequently develop severe PE and IUGR, and it is likely that these findings relate to the consequences of the pathophysiology of these disease. Further work is required to establish the nature and timing of the changes. It is encouraging that pregnancies at increased risk may be identified very early and this may help both to guide monitoring in clinical care and identify cases to investigate for intervention studies in research.

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