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Extracellular Nucleic Acids in Maternal Circulation as Potential Biomarkers for Placental Insufficiency

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Since the placenta is being continuously remodeled during normal placental development, extracellular nucleic acids of both fetal and placental origin, packed into either trophoblast-derived apoptotic bodies or shedding syncytiotrophoblast microparticles, may be detected in maternal circulation during the course of normal gestation. Placental-insufficiency-related pregnancy complications have been shown to be associated with excessive placental trophoblast apoptosis and shedding of placenta debris. Recent advances in the field are reviewed with a focus on the diagnostic potential of particular molecular biomarkers and their eventual implementation in the currently used predictive and diagnostic algorithms for placental-insufficiency-related pregnancy complications.

Introduction

Pre-eclampsia (PE) and intrauterine growth restriction (IUGR) are major complications affecting about 2%–5% of pregnancies responsible for maternal and perinatal morbidity and mortality (WHO, 1988). PE usually develops after 20 weeks of gestation and is characterized by chronic or gestational hypertension combined with proteinuria, which results from defective placentation, eliciting inadequate uteroplacental blood perfusion and ischemia (Khong *et al.*, 1986; ACOG Committee, 2002; Khan *et al.*, 2006). Recent evidence suggests that PE can be further subdivided into early PE (before 34 weeks of gestation), intermediate PE (between 34 and 37 weeks of gestation), and late PE (after 37 weeks of gestation) (Poon *et al.*, 2010a; Akolekar *et al.*, 2011). The concept of early and late PE is modern, and it is widely accepted that these two entities have different etiologies and should be regarded as different forms of the disease, where early onset of PE and IUGR are considered placenta-mediated diseases (Von Dadelszen *et al.*, 2003; Huppertz, 2008; Valensise *et al.*, 2008).

This differentiation makes it easier to distinguish, as early as possible, between those women at a higher, lower, and no risk for PE, optimally within 11–13 weeks of gestation, when prophylactic treatment may be considered and started (Poon *et al.*, 2010a; Akolekar *et al.*, 2011).

Currently, integrated algorithms combining maternal history and characteristics (maternal age, race, method of conception, smoking habits and substance abuse during pregnancy, history of chronic hypertension and type 1 or 2 diabetes mellitus, maternal family history of PE, obstetric history including parity, and previous occurrences of PE), biophysical tests (increased uterine artery pulsatility index and mean arterial pressure), and biochemical tests (altered maternal serum or plasma levels of relevant biochemical markers) at 11–13 weeks could potentially identify 90%, 80%, and 60% of pregnancies that subsequently develop early, intermediate, and late PE (5% rate of false positives) (Poon *et al.*, 2010a; Akolekar *et al.*, 2011; Beta *et al.*, 2011; Karagiannis *et al.*, 2011; Poon 2010b; Whitley 2007). Soluble endoglin (sEng), inhibin-A, activin-A, pentraxin-3 (PTX3), and P-selectin are increased, whereas serum pregnancy-associated plasma protein-A (PAPP-A), placental growth factor (PIGF), and placental protein-13 (PP13) are decreased in maternal circulation between 11 and 13 weeks of gestation in women who later develop PE (Poon *et al.*, 2009; Akolekar *et al.*, 2009, 2011). Unfortunately, none of these biochemical biomarkers are PE and/or IUGR specific. Using single biochemical markers usually has a low predictive value in the first trimester. PAPP-A, a glycoprotein synthesized in the placenta, has been shown to be a powerful predictive biochemical marker of PE when combined with Doppler ultrasound, reaching 70% sensitivity at 95% specificity (Spencer *et al.*, 2008; Goetzinger *et al.*, 2010; Anderson *et al.*, 2011). However, some recent studies evaluating PAPP-A as a first-trimester predictive marker for PE showed conflicting results that indicated no differentiation between complicated and uncomplicated pregnancies (Mikat *et al.*, 2011; Stirnemann *et al.*, 2011). PIGF, a potent angiogenic factor and soluble fms-like tyrosine kinase (sFlt-1), a soluble antiangiogenic vascular endothelial growth factor receptor (VEGFR), are promising biochemical markers that together show a high predictive power for PE, but no earlier than from the mid-second trimester. The prediction rate of the combination of decreased levels of PIGF and increased levels of sFlt-1 reached nearly 89% (De Vivo *et al.*, 2008; Anderson *et al.*, 2011). Hence, the

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sFlt-1/PIGF ratio has no predictive value in the first trimester. Low levels of PIGF itself have been shown to predict the early onset of PE with 53.5% sensitivity and a false-positive rate of 5% (Akolekar *et al.*, 2011). The development of automated methods has enabled the integration of PAPP-A, PIGF, and sFlt-1 biochemical markers into screening programs for PE (Verlohren *et al.*, 2010). With regard to other potential biochemical markers for placental-insufficiency-related pregnancy complications, more detailed research is necessary with a view toward routine assessment in everyday practice.

It has been clearly demonstrated that the establishment of a balance between trophoblast proliferation and apoptosis is crucial during normal placental development (Nelson, 1996). Both aging syncytiotrophoblasts and extravillous cytotrophoblasts undergo apoptosis (Oudejans *et al.*, 2003; Huppertz and Kingdom, 2004; Orozco *et al.*, 2006). Therefore, extracellular nucleic acids (DNA, messenger RNA [mRNA], and microRNAs [miRNA]) of both fetal and placental origin, packed into trophoblast-derived apoptotic bodies, may be detected in the maternal circulation during the normal course of gestation (Lo *et al.*, 1997). Size fractionation of circulatory DNA extracted from maternal plasma has revealed that apoptosis is followed by DNA degradation. The major proportion of circulatory fetal DNA fragments had an approximate molecular size of less than 300 bp (Li *et al.*, 2004; Chan *et al.*, 2004). Several recent studies have showed increasing levels of circulating nucleic acids (fetal DNA, placental-specific mRNA transcripts, and miRNAs) with advancing gestation, which reflects the growth of the placenta (Lo *et al.*, 1998; Ng *et al.*, 2003b; Hromadnikova *et al.*, 2011a, 2011b, 2011c, 2012). Additionally, the placenta sheds syncytiotrophoblast microparticles, which are also released into the maternal circulation (Reddy *et al.*, 2008).

The causes of PE and IUGR remain unknown. Nevertheless, a hypoxic environment induces excessive trophoblast cell death and increased shedding of placenta debris into the maternal circulation; as a result, placental-insufficiency-related pregnancy complications are associated with abnormal levels of extracellular fetal DNA and mRNA transcripts (Orozco *et al.*, 2006; Reddy *et al.*, 2008).

As the recent advances in the field are reviewed, there will be a focus on the diagnostic potential of particular molecular biomarkers and their implementation in the current predictive and diagnostic algorithms for placental-insufficiency-related pregnancy complications.

Extracellular DNA in Maternal Circulation

Detection of fetal-derived DNA in maternal circulation

Initially, researchers focused on the detection of male fetal-derived DNA in maternal circulation; most often, this was done using the single-copy sex-determining region Y (*SRY*) and/or multicopy *DYS-14* sequences on the Y chromosome, which are absent in the maternal genome (Lo *et al.*, 1997, 1998; Martinhago *et al.*, 2006).

Subsequently, Chan *et al.* introduced the *RASSF1A* sequence as a promising universal fetal DNA marker. The promoter of the *RASSF1A* gene turned out to be hypermethylated in the fetal part of the placenta and, therefore, resistant to methylation-sensitive restriction enzyme digestion, while maternally derived hypomethylated *RASSF1A* sequences were completely digested (Chan *et al.*, 2006).

Discrimination between normal pregnancies and those with placental-insufficiency-related pregnancy complications

Most quantification studies revealed higher concentrations of fetal DNA in placental-insufficiency-related complications (Table 1). Lo *et al.* (1999) showed a fivefold increase in the median concentration of extracellular fetal DNA, using real-time quantitative PCR assays for the *SRY* gene on the Y chromosome, in pre-eclamptic pregnancies (mean 32 weeks, range 27–41 weeks) compared with gestation age-matched controls). These data were later confirmed by other investigators using real-time quantitative PCR and either the *SRY* gene or the *DYS-14* sequence as markers to differentiate between normal and complicated pregnancies (Smid *et al.*, 2001; Zhong *et al.*, 2001; Lau *et al.*, 2002; Farina *et al.*, 2004b; Engel *et al.*, 2007; Alberry *et al.*, 2009; Hromadnikova *et al.*, 2009, 2010b). It was suggested that a rise in fetal DNA represented a valuable marker of placenta-related pregnancy complications, which could predict PE several weeks before clinical manifestation; however, with regard to IUGR, a rise in fetal DNA was less well correlated (Caramelli *et al.*, 2003; Sekizawa *et al.*, 2003; Farina *et al.*, 2004c; Zhong *et al.*, 2007; Hromadnikova *et al.*, 2010b).

Caramelli *et al.* (2003) demonstrated only a small increase in fetal DNA concentrations, determined using *SRY* TagMan PCR assays, in pregnancies with abnormal uterine artery Doppler waveforms that developed IUGR.

Sekizawa *et al.* (2003) demonstrated that fetal DNA concentrations were similar in both IUGR and normal subjects, determined using *DYS-14* sequence quantification in maternal plasma.

In 2007, Lo *et al.* patented the usage of hypermethylated *RASSF1A* sequences for monitoring fetal DNA in maternal plasma (Lo *et al.*, 2007). Subsequently, Tsui *et al.* (2007) reported a 4.3-fold higher concentration of hypermethylated *RASSF1A* sequences in the plasma of pre-eclamptic pregnancies compared with those observed in the controls (the onset time is not specified in the study). Later, Zhao *et al.* (2010) investigated the value of the hypermethylated *RASSF1A* sequence in maternal plasma within the third trimester of gestation (the onset time is not specified in the study) and found a positive correlation between its levels and the severity of PE.

The levels of total extracellular DNA, measured by quantifying the ubiquitous β-globin (*GLO*) and/or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) sequences in maternal circulation, were also found to be increased in this pathology (early/intermediate/late PEP, IUGR before and after 34 weeks) by several independent research groups (Zhong *et al.*, 2001; Sekizawa *et al.*, 2003) (Table 1).

Similarly, our previous studies revealed significantly increased levels of extracellular fetal and total DNA in pregnancies with PE with or without IUGR (median 34 weeks, range 26–40 weeks), relative to controls (using *SRY*, hypermethylated *RASSF1A* sequence, and β-*globin* as markers and real-time PCR) (Hromadnikova *et al.*, 2009, 2010b). While increased levels of extracellular DNA were detected in pregnancies with PE w/or w/o IUGR relative to controls (*RASSF1A* $p<0.001$; *SRY* $p=0.009$; and *GLO* $p<0.001$), quantities of fetal extracellular DNA in IUGR (median 28.5 weeks) were not statistically significant (*RASSF1A* $p=0.21$; *SRY* $p=0.2$). *RASSF1A*, *SRY*, and *GLO* achieved 93.1%,

Table 1. Summary of Extracellular DNA Biomarkers Evaluated to Differentiate Between Normal Pregnancies and Those with Placental Insufficiency

Placental insufficiency	*Marker*	*Reference*
SRY gene		
Onset	↑ PE	Lo *et al.* (1999), Zhong *et al.* (2001), Lau *et al.* (2002), Farina *et al.* (2004b), Engel *et al.* (2007)
	↑ PE ± IUGR	Smid *et al.* (2001), Hromadnikova *et al.* (2009), Hromadnikova *et al.* (2010b)
	Positive correlation between DNA levels and the severity of PE	Zhong *et al.* (2001), Engel *et al.* (2007)
	↑ IUGR	Smid *et al.* (2001)
	≅ IUGR	Hromadnikova *et al.* (2010b)
Before onset	↑ PE (17–18 weeks)	Leung *et al.* (2001)
	↑ PE (21 weeks)	Zhong *et al.* (2007)
	↑ IUGR (20–35 weeks) association with abnormal uterine artery Doppler waveforms	Caramelli *et al.* (2003)
	↑ PE and/or IUGR, but strongly individual (12–36 weeks)	Hromadnikova *et al.* (2010b)
	≅ IUGR (21 weeks)	Zhong *et al.* (2007)
DYS-14 sequence		
Onset	↑ PE	Alberry *et al.* (2009), Sekizawa *et al.* (2003)
	↑ PE ± IUGR	Hromadnikova *et al.* (2009)
	Positive correlation between DNA levels and the severity of PE	Alberry *et al.* (2009)
	↑ IUGR	Alberry *et al.*, 2009
	≅ IUGR	Sekizawa *et al.* (2003)
	Interindividual copy number variations influence quantification of extracellular fetal DNA	Hromadnikova *et al.* (2009)
Before onset	↑ early PE (11–13 weeks) association between DNA levels and uterine artery pulsatility index	Sifakis *et al.* (2009)
	↑ PE and/or IUGR before 35 weeks ≅ PE and/or IUGR after 35 weeks (11–14 weeks)	Illanes *et al.* (2009)
	↑ PE (18–21 weeks)	Farina *et al.* (2004c), Zhong *et al.* (2007)
	≅ IUGR (21 weeks)	Zhong *et al.* (2007)
DYS-1 sequence		
Before onset	↑ two-stage elevation in PE (17–28 weeks) and 3 weeks before onset of clinical symptoms	Levine *et al.* (2004)
Hypermethylated *RASSF1A* sequence		
Onset	↑ PE	Tsui *et al.* (2007), Zhao *et al.* (2010)
	↑ PE ± IUGR	Hromadnikova *et al.* (2010b)
	Positive correlation between DNA levels and the severity of PE	Zhao *et al.* (2010)
	≅ onset IUGR	Hromadnikova *et al.* (2010b)
Before onset	↑ PE and/or IUGR is strongly individual (12–36 weeks)	Hromadnikova *et al.* (2010b)
GLO gene		
Onset	↑ PE	Sekizawa *et al.* (2003)
	↑ PE ± IUGR	Hromadnikova *et al.* (2009), Hromadnikova *et al.* (2010b)
	↑ IUGR	Hromadnikova *et al.* (2010b)
Before onset	↑ PE and/or IUGR is strongly individual (12–36 weeks)	Hromadnikova *et al.* (2010b)
GAPDH gene		
Onset	↑ PE	Zhong *et al.* (2001)
	Positive correlation between DNA levels and the severity of PE	Zhong *et al.* (2001)
Before onset	≅ PE and/or IUGR (21 weeks)	Zhong *et al.* (2007)

↑ PE, extracellular DNA levels are increased in patients with PE; ≅ IUGR, extracellular DNA levels do not differ between controls and patients with IUGR; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *SRY*, sex-determining region Y; IUGR, intrauterine growth restriction; PE, pre-eclampsia.

93.6%, and 92.1% accuracy for differentiation between normal pregnancy and those with PE w/or w/o IUGR, respectively (Hromadnikova *et al.*, 2010b). Lower sensitivity was observed for all examined markers in pregnancies with the onset of IUGR (*RASSF1A*: 60.0%; *SRY*: 80.0%; and *GLO*: 72.7%), but it did not influence the final accuracy (*RASSF1A*: 91.6%; *SRY*: 92.5%; and *GLO*: 89.5%). Considerable variations in *DYS-14* copy numbers in men and discrepancies in *DYS-14* copy numbers between extracellular fetal DNA and the original fetal genome substantially limit the usage of the *DYS-14* sequence as a marker for extracellular fetal DNA quantification (Hromadnikova *et al.*, 2009).

Prediction of placental-insufficiency-related pregnancy complications

Leung *et al.* (2001) were the first who investigated whether an increase in circulating fetal DNA concentrations preceded the onset of clinical symptoms of PE (Table 1). The gestational age at the onset of clinical disease was 27.4–40 weeks. Although fetal DNA concentrations (assayed using real-time PCR) and the *SRY* gene as a marker were significantly higher at 17–18 weeks of gestation in pregnancies that later developed PE, the assay itself showed a relatively low sensitivity (67%) and specificity (82%) when used as the sole predictor for PE. Subsequently, Levine *et al.* (2004) observed, in a large-scale analysis involving 120 patients with PE and 120 controls, a two-stage elevation of cell-free fetal DNA in maternal sera before the onset of PE at 29–41 weeks of gestation. An initial elevation was seen between 17 and 28 weeks of gestation, and the second elevation was observed $\sim$3 weeks before the onset of clinical symptoms. The study was conducted using the TaqMan assay, which is specific for the *DYS1* gene used as a marker for the Y chromosome.

Later, Sifakis *et al.* (2009) demonstrated in another study, conducted at early gestational ages of 11–13 weeks, significantly higher levels of cell-free DNA in those women who subsequently developed early, but not late, PE, and an association between cell-free DNA levels and uterine artery pulsatility index. The study determined the amount of cell-free DNA using real-time PCR and the Y-chromosome *DYS-14* multicopy sequence.

The study performed by Illanes *et al.* (2009), in a similar manner (screening for *DYS-14* sequence in maternal circulation between 11 and 14 weeks), also found increased free fetal DNA levels in women who subsequently developed PE and/or IUGR and delivered before week 35 of gestation.

In 2009, Lo *et al.* patented the usage of the hypermethylated *RASSF1A* sequence for the monitoring of increased risk for developing PE (Lo *et al.*, 2009). Our study evaluated the quantification of fetal extracellular DNA in maternal plasma for the differentiation between cases at a risk of onset of placental-insufficiency-related complications and normal pregnancies (Hromadnikova *et al.*, 2010b). Using real-time PCR, fetal (*SRY* and hypermethylated *RASSF1A*) and total (*GLO* gene) extracellular DNA were examined in 18 pregnancies at a risk of placental-insufficiency-related pregnancy complications. Among the 18 patients at risk, 8 pregnancies involving 3 female and 5 male fetuses developed PE (1 early PE, 3 late PE), IUGR (1 case before 34 weeks, 2 cases after 34 weeks), and chronic placentopathy causing hypoxia ($n=1$). Elevation of extracellular DNA was demonstrated in 3 out of 5 (*SRY*), 1 out of 8 (hypermethylated *RASSF1A*), and 4 out of 8 (*GLO*) patients, ranging from 26 weeks, at the earliest, to 2 weeks before the onset of symptoms. While our data indicated an elevation of extracellular fetal and total DNA in patients at a risk of placental-insufficiency-associated pregnancy complications before the onset, this phenomenon was strongly individualized, could not be generalized to all cases, and was probably dependent on excessive placental trophoblast apoptosis. Interestingly, *SRY* and *GLO* quantification provided superior results compared with the hypermethylated *RASSF1A* sequence (Hromadnikova *et al.*, 2009, 2010b).

Extracellular mRNA in Maternal Circulation

The identification of reliable indicators of developing placental insufficiency and its related pregnancy complications is now feasible, thanks to the discovery of placental-specific mRNA transcripts in maternal circulation (Table 2).

mRNA transcripts from genes expressed in the placenta represent one group of promising new molecular biomarkers that can be used for all pregnancies, regardless of fetal gender and/or fetal genetic polymorphism. They are stable enough and readily detectable in maternal circulation throughout gestation. Therefore, the measurement of extracellular placental-specific mRNA biomarkers may enable regular noninvasive indirect monitoring of the growth, state, and functionality of the placenta. This strategy represents a significant step toward the future implementation of mRNA biomarkers in clinical diagnostic algorithms for following pregnancies at a risk of onset of placental-insufficiency-related pregnancy complications.

Detection of placental-specific mRNA in maternal circulation

In 2003, Ng *et al.* demonstrated the presence of mRNA of placental origin in maternal plasma, which provided other gender-independent markers for potential clinical use (Ng *et al.*, 2003b). mRNA transcripts from genes coding human placental lactogen (*hPL*) and the β subunit of human chorionic gonadotropin (β*hCG*) have been shown to be very stable in maternal plasma for up to 24 h at room temperature and rapidly cleared after delivery. *hPL* mRNA has been detected in all three trimesters of gestation, and β*hCG* mRNA has been observed in 100% cases through the first trimester, which indicates a correlation between plasma mRNA levels and the corresponding protein levels at various gestational stages. Farina *et al.* (2004a) demonstrated a positive correlation between corticotropin-releasing hormone (*CRH*) mRNA levels and gestational age in a retrospective study conducted on women with uncomplicated pregnancies between 27 and 40 weeks of gestation.

Discrimination between normal pregnancies and those with placental-insufficiency-related pregnancy complications

In 2003, Ng *et al.* also demonstrated 10.5 times higher concentrations of *CRH* mRNA in the plasma of women with late PE (median 37 weeks, range 36.6–38.9 weeks) compared with those from gestational age-matched control pregnancies (Ng *et al.*, 2003a). Farina *et al.* (2004a) confirmed the findings of Ng *et al.* and reported a positive correlation between circulating *CRH* mRNA and the severity of PE occurring

Table 2. Summary of Extracellular Messenger RNA Biomarkers Evaluated to Differentiate Between Normal Pregnancies and Those with Placental Insufficiency

Placental insufficiency	*Molecular biomarker*	*Reference*
CRH mRNA		
Onset	↑ PE	Ng *et al.* (2003a), Farina *et al.* (2004a), Purwosunu *et al.* (2007a), Galbiati *et al.* (2010)
	Positive correlation between mRNA levels and the severity of PE	Farina *et al.* (2004a); Purwosunu *et al.* (2007a)
	↑ IUGR	Galbiati *et al.* (2010)
Before onset	↑ PE (24–36 weeks)	Galbiati *et al.* (2010)
	≅ IUGR (32–36 weeks)	Galbiati *et al.* (2010)
GCM1 mRNA		
Onset	↑ PE	Fujito *et al.* (2006)
PLAC1 mRNA		
Onset	↑ PE	Fujito *et al.* (2006), Purwosunu *et al.* (2007a)
	Positive correlation between mRNA levels and the severity of PE	Purwosunu *et al.* (2007a)
Before onset	↑ PE (15–20 weeks) DR for PEP 17.7% at 5% FPR	Purwosunu *et al.* (2009)
hPL (*CSH1*) mRNA		
Onset	≅ PE	Fujito *et al.* (2006)
	↓ PE ± IUGR	Farina *et al.* (2006)
	≅ IUGR	Pang *et al.* (2009)
GH2 mRNA		
Onset	Positive correlation between mRNA levels and fetal growth (28–41 weeks)	Pang *et al.* (2009)
	≅ IUGR	Pang *et al.* (2009)
β*hCG* mRNA		
Onset	≅ PE	Fujito *et al.* (2006)
PAPP-A mRNA		
Onset	≅ PE	Fujito *et al.* (2006), Farina *et al.* (2006)
inhibin A mRNA		
Onset	↑ PE ± IUGR	Farina *et al.* (2006)
selectin P mRNA		
Onset	↑ PE ± IUGR	Farina *et al.* (2006)
	↑ PE	Purwosunu *et al.* (2007a)
	Positive correlation between mRNA levels and the severity of PE	Purwosunu *et al.* (2007a)
Before onset	↑ PE (15–20 weeks) DR for PE 24.2% at 5% FPR	Purwosunu *et al.* (2009)
VEGF mRNA		
Onset	↑ PE	Purwosunu *et al.* (2008)
	Positive correlation between mRNA levels and the severity of PE	Purwosunu *et al.* (2008)
Before onset	↑ PE (15–20 weeks) DR for PE 29% at 5% FPR	Purwosunu *et al.* (2009)
VEGFR-1 (*FLT1*) mRNA		
Onset	↑ PE ± IUGR	Farina *et al.* (2006)
	↑ PE	Purwosunu *et al.* (2008)
	Positive correlation between mRNA levels and the severity of PE	Purwosunu *et al.* (2008)
Before onset	↑ PE (15–20 weeks) the highest DR for PE (58%) at 5% FPR	Purwosunu *et al.* (2009)
KiSS-1 mRNA		
Onset	↓ PE ± IUGR	Farina *et al.* (2006)
	≅ IUGR	Pang *et al.* (2009)

(*continued*)

Table 2. (Continued)

Placental insufficiency	*Molecular biomarker*	*Reference*
PAI-1 (*SERPINE1*) mRNA		
Onset	↓ PE ± IUGR	Farina *et al.* (2006)
	↑ PE	Purwosunu *et al.* (2007b)
	Positive correlation between mRNA levels and the severity of PE	Purwosunu *et al.* (2007b)
Before onset	↑ PE (15–20 weeks)	Purwosunu *et al.* (2009)
	DR for PE 29% at 5% FPR	
tPA (*PLAT*) mRNA		
Onset	↑ PE	Purwosunu *et al.* (2007b)
	Positive correlation between mRNA levels and the severity of PE	Purwosunu *et al.* (2007b)
Before onset	↑ PE (15–20 weeks)	Purwosunu *et al.* (2009)
	DR for PE 33.9% at 5% FPR	
Endoglin mRNA		
Onset	↑ PE	Purwosunu *et al.* (2008)
	Positive correlation between mRNA levels and the severity of PE	Purwosunu *et al.* (2008)
Before onset	↑ PE (15–20 weeks)	Purwosunu *et al.* (2009)
	detection rate for PE DR 43.5% at 5% FPR	
ADAM12 mRNA		
Onset	↑ PE + IUGR	Pang *et al.* (2009)
PTX3 mRNA		
Before onset	↑ PE (24–36 weeks)	Galbiati *et al.* (2010)
	≅ IUGR (32–36 weeks)	Galbiati *et al.* (2010)

↑ PE, extracellular mRNA levels are increased in patients with PE; ≅ IUGR, extracellular mRNA levels do not differ between controls and patients with IUGR; β*hCG*, β subunit of human chorionic gonadotropin; *GCM1*, glial cells-missing 1; *CRH*, corticotropin-releasing hormone; *CSH1*, chorionic somatomammotropin hormone 1; *GH2*, growth hormone 2; *hPL*, human placental lactogen; mRNA, messenger RNA; *PAI*-1, plasminogen activator inhibitor type 1; PAPP-A, pregnancy-associated plasma protein-A; *PTX3*, pentraxin-3; *tPA*, tissue-type plasminogen activator; *VEGFR*, vascular endothelial growth factor receptor; DR, detection rate; FPR, false-positive rate; *PLAC1*, placenta-specific 1.

between 25.8 and 40 weeks of gestation (median 36.5 weeks). In 2006, Fujito *et al.* (2006) discovered a new promising noninvasive marker for PE (glial cells-missing 1 [GCM1]). They found increased plasma concentrations of placenta-specific 1 (*PLAC1*) and *GCM1* mRNAs in pre-eclamptic subjects than in controls (the onset time is not specified in the study). Concurrently, they observed no significant difference in *hPL*, β*hCG*, and *PAPP-A* mRNA levels between affected and unaffected patients. An independent study by Farina *et al.* (2006) tested a quantitative distribution of seven circulating mRNA markers chosen based on a previous gene expression study performed on placental tissues from normal and PE patients (median 33 weeks, range 27.7–37.4 weeks). This preliminary analysis revealed increased mRNA levels of inhibin A, selectin P, and *VEGFR* mRNAs and decreased mRNA levels of *hPL*, KiSS-1 metastasis-suppressor (*KISS-1*), and plasminogen activator inhibitor type 1 (*PAI-1*) in patients with early and/or intermediate PE with or without IUGR. Consistent with the study by Fujito *et al.*, no difference in *PAPP-A* mRNA levels was observed. However, Purwosunu *et al.* (2007b) reported significantly increased levels of both extracellular *PAI-1* and tissue-type plasminogen activator (*tPA*) mRNA transcripts in intermediate and late PE (median 39 weeks, range 35–41 weeks) and a positive correlation between both biomarkers and the severity of PE. tPA and its inhibitor PAI-1 may play a key role in fibrinolytic activity in the early stages of placentation and separation of the placenta from maternal tissue at term, as their expression was mainly concentrated at the interface where detachment from maternal tissue occurs (Hu *et al.*, 1999). Later, Purwosunu *et al.* confirmed preliminary data from their previous studies in a larger cohort of patients involving 43 intermediate/late PE (median 39 weeks, range 35–41 weeks) and 41 controls regarding the correlation of *CRH*, *PLAC1*, *selectin-P*, *VEGFR-1*, and *endoglin* mRNA plasma levels with the severity of PE and extended the panel for a new potential diagnostic marker, *VEGF*, which is a potent angiogenic factor involved in placental vascular development (Purwosunu *et al.*, 2007a, 2008). The study by Pang *et al.* (2009) evaluated whether circulating placental mRNAs could serve as markers for the assessment of fetal growth and IUGR from 28 to 41 weeks of gestation). They tested the panel of placental transcripts previously reported to be functionally involved in somatic growth, including chorionic somatomammotropin hormone 1 (*CSH1*), growth hormone 2 (*GH2*), *KISS1*, and ADAM metallopeptidase domain 12 (*ADAM12*). Only maternal plasma levels of *GH2* mRNA correlated with birth weight and fetal biometric measurements (fetal abdominal circumference, femur length, and biparietal diameter); however, they were not able to discriminate IUGR from normal pregnancies (Pang *et al.*, 2009).

Prediction of placental-insufficiency-related pregnancy complications

Meanwhile, the largest study was performed by Purwosunu *et al.* (2009), who examined, at gestational weeks 15–20,

62 patients who later developed PE (the onset time is not specified in the study) and 310 controls using the following panel of mRNA markers: plasminogen activator inhibitor-1 (*SERPINE1*), tissue-type plasminogen activator (*PLAT*), *VEGFA*, *VEGFA receptor 1* (*FLT1*), *endoglin*, *PLAC1*, and *selectin P*. Univariate analysis showed FLT1 as the marker with the highest detection rate (58%) and PLAC1 as the marker with the lowest detection rate (17.7%). The best multivariate model was obtained by the combination of all markers. A receiver operating characteristic curve yielded a detection rate of 84%, with a 5% false-positive rate, with an area under the curve of 0.927. This result offered the best prediction of PE in low-risk populations so far.

Further, Galbiati *et al.* (2010) evaluated a panel of protein (long PTX3 protein, PTX3), circulating DNA (fetal and total DNA based on methylation patterns of the *RASSF1A* promoter gene), and RNA (*CRH* and *PTX3* mRNAs) as potential markers in a group of 52 women at a risk of placental-insufficiency-related pregnancy complications based on a history of these pathologies in previous pregnancies. Three patients from the studied cohort developed PE (two early PE at 28 weeks, one postpartum PE), and six of them developed IUGR between 28 and 36 weeks of gestation. Several weeks before the onset of PE, significant increases in fetal and total DNA levels and *CRH* mRNA levels were observed; however, before the onset of IUGR, only increased total extracellular DNA levels were found.

Placental-Specific miRNAs as Promising Biomarkers of Placental Insufficiency

Nevertheless, recent studies have offered the possibility of exploiting a new class of molecular markers, miRNAs, for the diagnosis and prediction of pregnancy-related complications, such as PE and IUGR. miRNA analyses indicate that diverse affected tissues display miRNA expression profiles to be significantly different from normal tissues, which may be crucial in a wide range of clinical diagnostic applications (Calin and Croce, 2006; Rosenfeld *et al.*, 2008). Another reason for the assessment of the role of miRNAs in placental insufficiency is that they are believed to be critical in cell development, proliferation, communication, and death, all of which are significantly altered in PE and/or IUGR (Baehrecke, 2003; Brennecke *et al.*, 2003; Bartel *et al.*, 2004; Ason *et al.*, 2006; Kloosterman and Plasterk, 2006).

miRNAs belong to a family of small noncoding RNAs (18–25 nucleotides) that regulate gene expression at the post-transcriptional level by degrading or blocking the translation of mRNA targets (Lai, 2002; Bartel, 2004).

Briefly, miRNAs are mostly synthesized as an miRNA/miRNA* duplex. One of the duplex strands is referred to as mature miRNA, and its counterpart, which is degraded after release of the mature strand from the duplex, is called miRNA* (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003).

Differential expression of miRNAs between pre-eclamptic and control placentas

Recent research has shown that miRNAs are abundantly expressed in the human placenta (Liang *et al.*, 2007; Mayor-Lynn *et al.*, 2011). Pineles *et al.* (2007) was the first who reported the differential expression of *miR-210* and *miR-182* between placentas of pre-eclamptic patients and controls (both miRNAs were up-regulated in PE, the onset time is not specified in the study). Zhu *et al.* (2009) performed a comprehensive analysis of 455 miRNA expression profiles in pre-eclamptic versus normal placentas between 36 and 40 weeks of gestation and found 11 miRNAs (*miR-181a*, *miR-584*, *miR-30a-3p*, *miR-210*, *miR-152*, *miR-517*, *miR-518b*, *miR-519e*, *miR-638*, *miR-296*, and *miR-362*) to be overexpressed and another 23 to be underexpressed (*miR-101*, *miR-10b*, *miR-218*, *miR-590*, *miR-32*, *miR-204*, *miR-126*, *miR-18a*, *miR-19a*, *miR-411*, *miR-377*, *miR-154*, *miR-625*, *miR-144*, *miR-195*, *miR-150*, *miR-1*, *miR-18b*, *miR-363*, *miR-542-3p*, *miR-450*, *miR-223*, and *miR-374*) in pre-eclamptic patients. Hu *et al.* (2009) performed a high-throughput miRNA microarray study and revealed significantly increased levels of 7 miRNAs (*miR-16*, *miR-29b*, *miR-195*, *miR-26b*, *miR-181a*, *miR-335*, and *miR-222*) in placentas derived from pregnancies with severe PE, occurring after 34 weeks, compared with those derived from healthy controls. Mayor-Lynn *et al.* (2011) focused on microarray profiling of 820 miRNAs and 18,630 mRNA transcripts in placentas and found different expressions of 20 miRNAs and 120 mRNAs in the placentas from patients with PE sampled between 31 and 39 weeks (median 35 weeks) and spontaneous preterm labor at $\leq$35 weeks of gestation (median 28 weeks) compared with normal term pregnancies (median 38.1 weeks). Confirmation experiments of selected miRNAs using real-time PCR indicated lower expression of *miR-15b*, *miR-181*, *miR-210*, and *miR-483-5p* in placentas affected by PE and preterm labor. Consistent with Pineles *et al.*, Zhu *et al.*, and Zhang *et al.*, but inconsistent with Mayor-Lynn *et al.*, the study by Enquobahrie *et al.* found *miR-210* to be up-regulated among late-onset PE cases (mean 36 weeks) compared with controls (mean 38.8 weeks) (Zhu *et al.*, 2009; Enquobahrie *et al.*, 2011; Mayor-Lynn *et al.*, 2011; Zhang *et al.*, 2011). Enquobahrie *et al.* (2011) further confirmed the downregulation of previously identified miRNA candidates (*miR-1*, *miR-328*, *miR-139-5p*, *miR-500*, and *miR-1247*) and found two novel miRNA candidates to be downregulated in pregnancies complicated by PE (*miR-584* and *miR-34c-5p*). Noack *et al.* (2011) identified another six miRNAs to be overexpressed in five cases with severe PE who delivered at gestational age ranging from 29 to 37 weeks (*let-7b*, *miR-302**, *miR-104*, *miR-128a*, *miR-182**, and *miR-133b*).

However, application of these findings to routine practice requires monitoring of extracellular miRNAs in the maternal circulation.

Detection of placental-specific miRNAs in maternal circulation

In 2008, Gilad *et al.* (2008) reported that serum miRNAs might be used as promising new biomarkers for differentiation between pregnant and nonpregnant women.

Chim *et al.* (2008) identified 17 out of 157 miRNAs in significantly higher concentrations in normal placentas than in maternal blood cells and the 4 most abundant placental miRNAs (*miR-141*, *miR-149*, *miR-299-5p*, and *miR-135b*) in maternal plasma samples. Similarly, in another study, Miura *et al.* (2010) identified 24 pregnancy-associated miRNAs. However, the investigators finally selected, for further analysis, only 5 miRNAs (*miR-515-3p*, *miR-517a*, *miR-517c*, *miR-518b*, and *miR-526b*) that showed a significant increase in maternal plasma concentrations through gestation and a significant decrease after pregnancy termination.

The main goal of our concurrently running study was to identify placental specific miRNAs with a plasma expression profile that would differ significantly between normal pregnancies and placental-insufficiency-complicated pregnancies (Hromadnikova *et al.*, 2010a; Kotlabova *et al.*, 2011). Initially, we tested 20 miRNAs that had been selected on the basis of two previous findings, placenta specificity according to the miRNAMap database and a study presented by Liang and colleagues (Liang *et al.*, 2007; Chim *et al.*, 2008; Zhu *et al.*, 2009). The selection of appropriate miRNAs with a diagnostic potential was based on the following criteria: (1) a detection rate of 100% in full-term placentas and maternal plasma throughout gestation and (2) a detection rate of 0% in whole peripheral blood and plasma samples of nonpregnant women. Seven miRNAs (*miR-516-5p*, *miR-517**, *miR-518b*, *miR-520a**, *miR-520h*, *miR-525*, and *miR-526a*) have been recently identified as pregnancy-associated ones with a diagnostic potential (Kotlabova *et al.*, 2011).

Discrimination between normal pregnancies and those with placental insufficiency

The aims of our consecutive pilot study were to quantify placental-specific miRNAs (*miR-516-5p*, *miR-517**, *miR-518b*, *miR-520a**, *miR-520h*, *miR-525*, and *miR-526a*) in maternal circulation in normal pregnancies, to determine whether they could differentiate between pregnancies with the onset of placental-insufficiency-related complications and normally progressing pregnancies, and to determine whether they are able to differentiate, during the early stages of gestation, between normal pregnancies and pregnancies at a risk of developing PE and/or IUGR (Hromadnikova *et al.*, 2011a, 2011b, 2011c, 2012). Absolute and relative quantification of placental-specific miRNAs (*miR-516-5p*, *miR-517**, *miR-518b*, *miR-520a**, *miR-520h*, *miR-525*, and *miR-526a*) were determined in 50 normal pregnancies, 32 complicated pregnancies (20 cases with early onset before 34 weeks and 12 cases with late onset after 34 weeks), and 7 pregnancies at various gestational stages that later developed PE and/or IUGR (3 before 34 weeks and 4 after 34 weeks of gestation) using real-time PCR and a comparative Ct method relative to normalization factor (geometric mean of ubiquitous *miR-16* and *let-7d*).

Both quantification approaches revealed significant increases in extracellular placental-specific miRNAs levels over time in normally progressing pregnancies; however, they were not able to differentiate between normal and complicated pregnancies at the time of PE and/or IUGR onset. Nevertheless, a significant elevation in extracellular miRNAs was observed during early gestation (from 12 to 16 weeks) in pregnancies with an onset of PE and/or IUGR.

This phenomenon might reflect the temporarily increased expression of particular miRNAs in placentas with incipient placental insufficiency. It could also be explained by the transiently enhanced apoptosis of extravillous trophoblasts between 11 and 16 weeks of gestation, accompanied by an increase in extracellular placental-specific miRNAs levels in maternal circulation that had been subsequently normalized (Cotter *et al.*, 2004). Based on the results of this pilot study, a large-scale analysis was initiated. The panel of selected extracellular miRNAs is being validated for implementation in the first trimester screening to identify high-risk pregnancies. These data strongly support the need for a more detailed exploration of extracellular miRNAs in maternal circulation with the view toward routine assessment as a part of everyday clinical practice, and recognition as a potential biomarker for placental-insufficiency-related complications.

Prevention of PE and IUGR

It was thought that low-dose aspirin could inhibit thromboxane-mediated vasoconstriction, thereby protecting against vasoconstriction and pathological blood coagulation in the placenta (Thorp *et al.*, 1988; Bujold *et al.*, 2010).

To estimate the effect of low-dose aspirin therapy (50–150 mg of acetylsalicylic acid daily) on the incidence of PE and IUGR, a systemic review and meta-analysis were performed through electronic database searches (Bujold *et al.*, 2010). The study revealed that low-dose aspirin therapy started before 16 weeks of gestation was associated with a significant reduction in the incidence and severity of PE, IUGR, and preterm birth (before 34 weeks of gestation) in women identified to be at a moderate or high risk for PE using various inclusion criteria, mostly anamnestic maternal risk factors and eventually abnormal uterine artery Doppler velocimetry (Bujold *et al.*, 2010). However, the latest longitudinal study carried out on the cohort of 6437 children at 12 years of age, whose mothers used aspirin most days or daily during pregnancy, revealed an association between maternal analgesic use during pregnancy and a risk of psychotic syndromes during adolescence (Gunawardana *et al.*, 2011).

Concluding Remarks

In conclusion, numerous methods have attempted to predict placental-insufficiency-related pregnancy complications using biochemical and/or molecular biomarkers. However, the best sensitivity and specificity are obtained when these tests are performed at the end of the second trimester.

There is a need for exploration of the first-trimester biomarkers that would enable the identification of patients at a risk of early onset PE and/or IUGR (i.e., before 34 week of gestation), which is associated with a higher incidence of maternal and perinatal morbidity and mortality.

Two independent studies reported early elevated levels of extracellular fetal DNA in patients who subsequently developed severe PE and/or IUGR (Illanes *et al.*, 2009; Sifakis *et al.*, 2009). However, both studies screened the levels of male fetal DNA in maternal circulation by using the *DYS-14* sequence as a marker, with interindividual copy number variations that were later proved to influence extracellular fetal DNA quantification (Hromadnikova *et al.*, 2009). Further work is required to evaluate the utility of single-copy *SRY* and hypermethylated *RASSF1A* sequences for extracellular fetal DNA quantification, during the first trimester of gestation, to differentiate early- and late-onset PE and/or IUGR from normally progressing pregnancies.

Several studies have reported an association between increased circulating levels of numerous mRNA transcripts and an augmented risk for subsequent development of placental-insufficiency-related pregnancy complications. However, these studies focused on the examination of extracellular mRNA distribution among women with PE and/or IUGR and control subjects mainly between 15 and 36 weeks of gestation. It might be worthwhile to undertake a

systematic investigation of extracellular placental-specific gene expression targeted to the first trimester of gestation and correlate it with the severity of the disease with regard to not only the degree of clinical signs (ACOG guidelines), but also the period of onset of the disease and requirements for delivery (before and after 34 weeks of gestation).

Currently, the extracellular placental-specific miRNAs assessed seem to be promising biomarkers and should be explored in further large-scale studies to confirm their ability to differentiate between normal and complicated pregnancies in the early weeks of gestation.

Finally, the approach of extracellular nucleic acids quantification might enable clinicians to monitor, noninvasively, pathophysiological alterations in the placenta at any time during the course of gestation. Since changes in the placenta start in the first trimester, this strategy might also help identify women with placental insufficiency as early as possible, and start preventive measures at a far earlier stage of gestation, at least in patients at a high risk for early-onset PE and/or IUGR.

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The authors declare no conflict of interest, and no competing financial interests exist.

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