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MATERNAL SERUM BIOCHEMICAL MARKERS PP13, PAPP-A, PlGF AND ADAM12 @ 11-13^{+6} WEEKS' GESTATION AND ADVERSE PREGNANCY OUTCOMES

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submitted by
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to my family
and
Alwis Cramer

Contents

1. Introduction

Abnormal placentation can lead to adverse pregnancy outcomes such as small for gestational age (SGA), pre-eclampsia (PE) and hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, contributing to fetal and maternal morbidity and mortality. PE affects 3-5% of all pregnancies (Roberts and Cooper, 2001), and in its most severe form is also associated with SGA and requires induced early delivery. HELLP is a multisystemic disease severely affecting both the mother and the fetus which can be found in 0.17-0.85% of all pregnancies and appearing in 20% of severe pre-eclamptic cases. The criteria for the diagnosis of the condition are focused on markers for hemolysis, liver enzymes and thrombocytopenia (Mihu et al., 2007; Sibai and Ramadan, 1993).

There are currently no effective preventive therapies for these disorders, and the only treatment for PE is induced delivery. Despite this, predictive markers would still be of value by identifying high risk pregnancies for future potential interventional trials as well as allowing additional monitoring of these pregnancies.

More recently however, a metaanalysis of nine randomized controlled trials enclosing 1317 women with PE has assessed the influence of gestational age at the time of introduction of Aspirin. Aspirin treatment beginning in early gestation was associated with a greater reduction in the incidence of preeclampsia than treatment beginning in late gestation: When Aspirin treatment started at 16 weeks' gestation or below the RR was 0.48 (95% CI 0.33 to 0.68), at 17-19 weeks' 0.55 (95% CI 0.17 to 1.76) and at 20 weeks' or higher 0.82 (95% CI 0.62 to 1.09). When Aspirin treatment started especially before 16 weeks there was also a significant reduction in the incidence of severe preeclampsia (RR 0.10; 95% CI 0.01 to 0.74), gestational hypertension (RR 0.31; 95% CI 0.13 to 0.78) and IUGR (RR 0.51; 95% CI 0.28 to 0.92) (Bujold et al., 2009).

Ultrasound uterine artery Doppler provides information on the maternal blood flow to the placenta. Second trimester Doppler has been demonstrated to detect 45% - 55% of PE pregnancies (at 5% false positive rate (FPR)) increasing to 62% in combination with first trimester PAPP-A (Papageorghiou et al., 2004; Spencer et al., 2005). It is likely that first or second trimester biochemical markers such as PP13, PAPP-A, Inhibin and Activin A, in combination with Doppler and maternal biophysical parameters such as blood pressure or arterial stiffness, can increase the sensitivity (Spencer et al., 2005; Spencer et al., 2007a; Spencer et al., 2008c, Poon et al., 2010, Khalil et al., 2009a). Various biomarkers are under investigation for both PE and SGA.

PP13 is a 32 KD dimer belonging to the galectin family of proteins (Than et al., 2004). It is expressed in placenta and it has been shown to play a role in trophoblast implantation, invasion and development of placenta during early stages of gestation (Than et al., 2004; Burger et al., 2004). Molecular studies on PP13 mRNA expression on trophoblasts of women with pre-eclamspia, have shown a down-regulation of the gene during first and third trimester (Sekizawa et al., 2008; Than et al., 2008). PP13 levels have been shown to be decreased in PE and SGA (Than et al., 2004; Chafetz et al., 2007; Gonen et al, 2008; Huppertz et al., 2008; Khalil et al,. 2009b; Nicolaides et al. 2006; Romero et al., 2008; Spencer et al., 2007a; Spencer et al., 2007b). In one published study, PP13 levels were not found to be significantly different in SGA pregnancies (Cowens et al., 2008). This is the first reported study using the new AutoDELFIA assay to measure PP13.

PAPP-A belongs to the metzincin superfamily of zinc metalloproteases and is a regulator of IGF bioactivity in several systems (Lawrence et al., 1999). Through cleavage, PAPP-A causes a reduction in the affinity of insulin-like growth factor binding protein-4 (IGFBP-4) to IGF-I and II. Thus, PAPP-A has been proposed as a potential marker for dysfunctions of placental development such as SGA (Canini et al., 2008; Cowans et al., 2007; Spencer et

al., 2008a) and preeclampsia (Spencer et al., 2005; Pilas et al., 2007; Poon et al., 2009; Spencer et al., 2008b).

Placenta growth factor (PlGF) is a dimeric glycoprotein member of the angiogenic vascular endothelial growth factor family, first isolated in 1991 and later found to be expressed predominantly by trophoblasts, and is able to cause endothelial cell proliferation, migration and activation (Maglione et al., 1991; Shore et al., 1997; Vuorela et al., 1997). In pregnancy, it is believed that PlGF and other angiogenic factors regulate trophoblast invasion of the maternal spiral arteries. Impaired trophoblast invasion leads to insufficient vascular remodelling of the spiral arteries than that which occurs in normal pregnancy. This leads to reduced perfusion of the placenta which is conceivably involved in the following adverse pregnancy outcomes: small for gestational age (SGA) pre-eclampsia (PE) and haemolysis elevated liver enzymes and low platelets (HELLP) (Weinstein 1982; Barker et al., 1993; Sibai and Ramadan 1993; Curtin and Weinstein 1999; Dekker and Sibai 2001; Roberts and Cooper 2001; Sibai et al., 2005; Mihu et al., 2007). Several studies of pregnancies affected by preeclampsia have indicated that PlGF levels are decreased in the first and second trimesters, as well as at the stage of clinical onset of the disease (Torry et al., 1998; Reuvekamp et al., 1999; Tjoa et al., 2001; Tidwell et al., 2001; Su et al., 2001; Taylor et al., 2003; Polliotti et al., 2003; Akolekar et al., 2008; Crispi et al., 2008). Studies in the literature have shown inconsistent first trimester PlGF patterns in pregnancies resulting in SGA (Ong et al., 2001; Thadhani et al., 2004; Smith et al., 2007; Poon et al., 2008a; Erez et al., 2008).

A-disintegrin-and-metalloprotease-12 (ADAM12) is a multidomain glycoprotein belonging to the reprolysin zinc metalloproteases family (Loechel et al., 1998). Alternative splicing produces a long and short form, and the short, circulating form has been shown to have cell adhesion property and proteolytic activity against binding proteins for insulin-like growth factor (Shi et al., 2000; Loechel et al., 2000). When first investigated in the

maternal circulation using a small series of samples on a quantitative enzyme-linked immunosorbent assay (ELISA), ADAM12 appeared to be an extremely promising marker for chromosomal aneuploides, with markedly reduced levels of the molecule in affected first trimester trisomies 21 and 18 maternal serum (Laigaard et al., 2003; Laigaard et al., 2005a). Later studies, using a new DELFIA platform (PerkinElmer Life Sciences, Turku, Finland), supported these early results, placing the discriminatory power of ADAM12 greatest prior to 10 weeks gestation (Laigaard et al., 2006a; Laigaard et al., 2006b; Spencer et al., 2007c; Spencer et al., 2008d,e). In the second trimester, ADAM12 levels were found to be increased in trisomy 21 pregnancies (Christiansen et al., 2007; Donalson et al., 2008). As a marker for adverse pregnancy outcomes, ADAM12 has been shown to be decreased in first trimester maternal serum of pregnancies which later went on to develop PE, and even further decreased in pregnancies which also resulted in a low birth weigh delivery (Laigaard et al., 2005b), or early, severe PE (Laigaard et al., 2005b; Spencer et al., 2008f). In addition, ADAM12 levels have been shown to decrease in pregnancies which resulted in growth restricted fetuses, supporting a role of ADAM12 in fetal growth (Cowans and Spencer, 2007; Pihl et al., 2008). However, a recent study has found no significant difference in first trimester maternal serum ADAM12 concentrations between controls and pregnancies resulting in PE (Poon et al., 2008b). In addition, although this study did find a decrease in first trimester maternal serum ADAM12 concentrations in IUGR pregnancies, ADAM12 failed to emerge as a successful marker for this adverse outcome. To date, there are no published studies looking at ADAM12 and HELLP syndrome. The aim of the current study is to evaluate further the role of ADAM12 as a potential marker of various adverse pregnancy conditions, including HELLP.

In this case control study we aim to investigate first trimester maternal serum PP13, PAPP-A, PlGF and ADAM12 levels in a number of pregnancies which later developed one

or more of the following adverse outcomes: PE, IUGR, HELLP and GH. The rationale for the development of screening for PE at 11-13^{+6} weeks' gestation is the much higher risk reduction if Aspirine treatment is started early.

2. Methods

2.1 Study population

This is a case-control study, part of a larger study supported by the European Union FP6 (Pregenesys, Grant No. 037244) looking at first-trimester biochemical marker levels in pregnancies resulting in adverse outcomes. Maternal serum samples were collected at the University Hospital Schleswig-Holstein, Kiel, Germany, as part of an 11 +0 to 13 +6 weeks' screening program for risk detection of fetal abnormalities. Gestational age was determined from the known date of the last menstrual period (LMP) and recalculated by ultrasonographic measurement of the crown-rump length (CRL) when exceeding more than 7 days' difference to the last menstrual period. Simultaneously, the nuchal translucency measurement was taken. Additionally, maternal serum free β-hCG and PAPP-A were measured using Kryptor (Brahms AG, Hennigsdorf, Germany). All components of the scan and the laboratory were licensed through the Fetal Medicine Foundation, London, UK. The parents were counselled immediately after the examination. Maternal demographic data (age, ethnic origin, smoking status, parity, height and weight) were recorded at the time of sampling and stored in a database. Informed consent was given for the use of excess serum, which was approved by the Ethical Committee of the Christian Albrecht University of Kiel, Kiel, Germany.

Samples were collected between October 1998 and April 2008 and stored frozen until analysis (the median duration of storage was 5.17 years). Samples were generally stored at –70 ° C; however, some were stored intermittently at –20 ° C for up to 4 weeks in Germany and were shipped on ice by car to the UK in two batches (in 2004 and 2008) where they were stored at –20 ° C until testing.

During the 10-year period of collection time, 3040 pregnancies were screened and delivered at this centre. From manual evaluation of labour ward books looking for written

evidence of PE, HELLP, GH and SGA, 56 abnormal pregnancies were identified and confirmed by accessing patient and birth records. Nine of these were excluded due to serum being unavailable. Many cases, in particular SGA, may have been missed when it was not recorded in the labour ward records, and logistically it was not feasible to search all birth records by birth weight. For each of the 47 cases, 10 chromosomally and structurally normal pregnancies which did not develop any adverse outcomes were selected for controls, matched for storage time and gestational age with the cases. 18 of these were excluded due to serum being unavailable.

Figure 1 shows the number of controls and cases of *all PE* , *all SGA* and *GH* , as well as subsets created from these groups for *HELLP* , *early* and *late PE* and *SGA* (onset or delivery before or after 34 weeks), *PE and SGA* , *PE only* (without SGA nor HELLP) and *SGA only* (without PE).

The samples were also measured for Placental Protein 13 (PP13), placental growth factor (PlGF) and 'A disintegrin and metalloprotease-12' (ADAM12) (Cowans et al., 2007; 2008; 2010; Matwejew et al., 2010, Stamatopoulou et al., 2010).

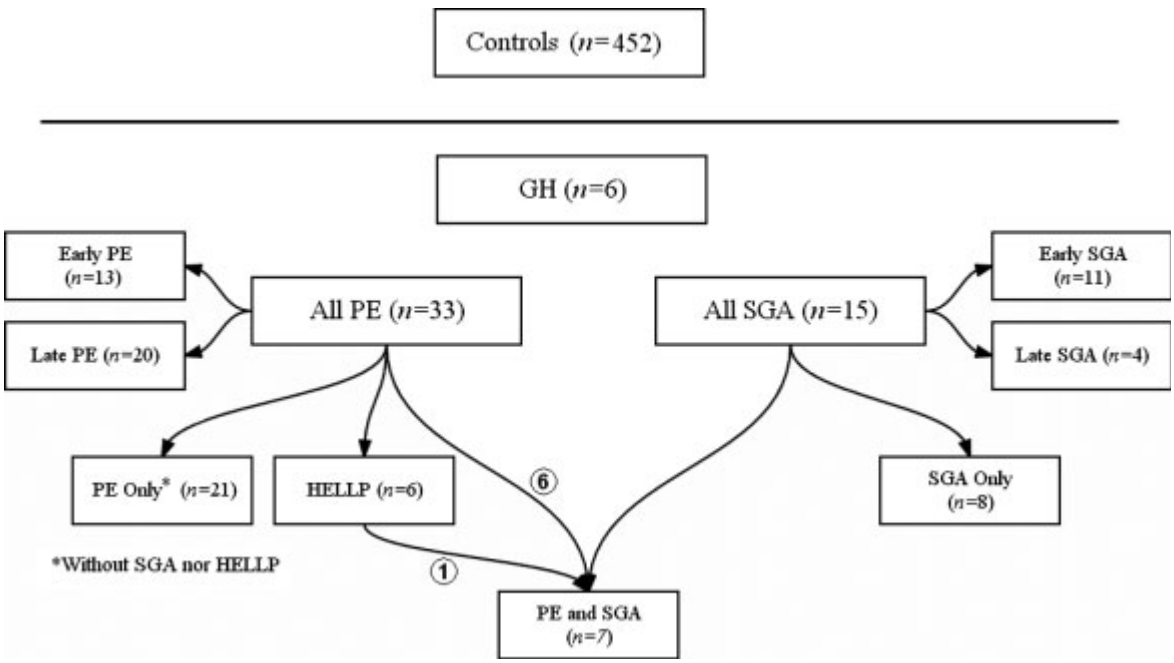

Figure 1. Case (and subsets) and control sample numbers.

2.1.1 Inclusion criteria

All cases screened at the University of Schleswig-Holstein, Campus Kiel, Department of Obstetrics and Gynecology, Germany, collected between October 1998 and August 2008, from 11-13^{+6} weeks gestation, including both a measurement of nuchal translucency and first trimester serumbiochemistry.

2.1.2 Exclusion criteria

Multiple gestation, fetal structural or chromosomal abnormalities, fetal death, incomplete or missing screening at 11-13^{+6} weeks gestation.

2.2 Adverse outcomes

The definitions of PE and GH were those of the International Society for the Study of Hypertension in Pregnancy (Davey et al., 1988). GH is defined as diastolic blood pressure ≥ 90 mmHg on two or more occasions, at least 4 hours apart, appearing after 20 weeks gestation in a previously normotensive woman, in the absence of significant proteinuria. PE is GH with ≥ 300 mg proteinuria in 24 hours, or two readings of at least ++ on dipstick analysis of midstream or catheter urine specimens in the absence of 24-hour collection availability.

SGA was defined as a birth weight below the tenth percentile for the individual birth gestational day, using the Hadlock method and ViewPoint growth charts (Marsal et al., 1996).

2.3 Pre-analytical sample processing

Samples were analyzed at the Department of Obstetrics and Gynecology, Christian Albrechts University at Kiel (free beta hCG and PAPP-A) and at the Department of Clinical

Biochemistry King George Hospital Barley Lane Goodmayes, London, UK (PP13, PlGF and ADAM12).

Maternal venous blood was taken from the antecubital vein at the time of the nuchal scan (11-13^{+6} weeks). Monovette® test tubes (Neutral or Serum Z/7.5 ml, SARSTEDT, Sarstedt AG & Co, Nümbrecht, Germany) were used without any anticoagulants to obtain blood serum. Blood was spun within 30 minutes after it was taken and frozen (1.5ml Eppendorf tubes, no anticoagulants, Hamburg, Germany). All serum samples in each group were stored at -70°C in two to four aliquots. Samples were kept frozen at all times. Free beta hCG and PAPP-A was immediately analyzed at the time of screening. Samples were transferred to the Department of Clinical Biochemistry King George Hospital Barley Lane Goodmayes, London, UK, and kept frozen at all times. One to two aliquots were thawed, spun and the supernatant used for further analysis. Immediately before the final measurement samples were visually checked for hemolysis, hyperbilirubinemia and lipemia. All samples which were not perfectly clear were recorded. Biotin levels were not considered.

2.4. Sample analysis

2.4.1 PP13

The samples were analysed for PP13 concentrations by automated time resolved fluoroimmunoassays using the AutoDelfia platform (PerkinElmer Life Science, Turku, Finland). Briefly, the method is a solid-phase, two-site fluorometric sandwich assay where monoclonal antibodies are directed against two separate antigenic determinants on PP13. The tracer antibody is europium-labelled, from which Eu-N3 chelate complexes form after addition of DELFIA inducer solution, which are counted by the AutoDelfia counter. PP13 standards and samples were run in duplicate. Lab constructed serum controls were run in singleton at the start and end of each plate in all assays.

The analytical range of the assay is 14 to 861 pg/ml. Samples were run blind to outcome.

2.4.2 PAPP-A

PAPP-A concentrations were determined as part of the first trimester screening program patients had enrolled in, by Kryptor analyser (Brahms AG, Berlin) as described previously (Spencer et al., 1999).

2.4.3 PlGF

The samples were analysed for PlGF concentrations by an automated time resolved fluoroimmunoassay using the AutoDelfia platform (PerkinElmer Life Science, Turku, Finland). Briefly, the method is a solid-phase, two-site fluorometric sandwich assay where monoclonal antibodies are directed against two separate antigenic determinants on PlGF. The tracer antibody is europium-labelled, from which Eu-N3 chelate complexes form after addition of DELFIA inducer solution, which are measured by the AutoDelfia fluorescence reader. PlGF standards were run in duplicates while samples were run in singleton. Lab constructed serum controls were run in singleton at the start and end of each plate in all assays.

The assay range is 10 to 999 pg/ml, and the samples were analysed without knowledge of the clinical outcome.

2.4.4 ADAM12

The samples were analysed for ADAM12 concentrations by an automated time resolved fluoroimmunoassay using the AutoDelfia platform (PerkinElmer Life Science, Turku, Finland). Briefly, the method is a solid-phase, two-site fluorometric sandwich assay where monoclonal antibodies are directed against two separate antigenic determinants on ADAM12. The tracer antibody is europium-labelled, from which Eu-N3 chelate complexes

form after addition of DELFIA inducer solution, which are measured by the AutoDelfia fluorescence reader. ADAM12 standards were run in duplicates while samples were run in singleton. Lab constructed serum controls were run in singleton at the start and end of each plate in all assays.

The assay range is 15 to 1518 ng/ml, and the analytical sensitivity of the assay was typically >6ng/ml. The samples were analysed without knowledge of the clinical outcome.

2.5 Statistics

2.5.1 PP13

PP13 levels were converted to multiples of the median (MoM) by dividing each individual result by the expected median marker of the control group at that gestational age (day). The control group was clustered into 10kg maternal weight bands, and the median $\log_{10}$ PP13 MoM for each cluster was plotted against the median weight for each cluster to derive an equation to determine the weight corrected MoM, as outline previously (Spencer et al., 2003). The population was of the same ethnic origin, and PP13 levels were corrected for smoking status.

2.5.2 PAPP-A

PAPP-A MoMs were determined using ViewPoint at the time of screening, corrected for maternal weight and smoking status. Statistical significance was determined using Mann-Whitney test for continuous variables, and $\chi 2$ test for categorical variables.

2.5.3 PlGF

PlGF levels were converted to multiples of the median (MoM) by dividing each individual result by the expected median marker of the control group at that gestational age. The control group was clustered into 10kg maternal weight bands, and the median log10 PlGF MoM for each cluster was plotted against the median weight for each cluster to determine

if correction was required with PlGF (Spencer et al., 2003). The population was of the same ethnic origin (Caucasian), and PlGF levels were corrected for smoking status.

Statistical significance was determined using Mann-Whitney test for continuous variables, and $\chi 2$ test for categorical variables.

2.5.4 ADAM12

ADAM12 levels were converted to multiples of the median (MoM) by dividing each individual result by the expected median marker of the control group at that gestational age. To account for haemodilution, the control group was clustered into 10kg maternal weight bands, and the median log10 ADAM12 MoM for each cluster was plotted against the median weight for each cluster to derive an equation to determine the weight corrected MoM, as outline previously (Spencer et al., 2003). The population was of the same ethnic origin (Caucasian), and ADAM12 levels were corrected for smoking status.

Statistical significance was determined using Mann-Whitney test for continuous variables, and $\chi 2$ test for categorical variables.

3. Results

3.1 PP13

The demographic data for gestational age (weeks), maternal age (years), smoking, parity and maternal weigth (kg) for the controls, early onset and late onset PE, PE±SGA, HELLP Syndrome and GH are shown (Table 1).

In the control group, PP13 concentrations increased slightly with gestational age, with median PP13 values for 11, 12 and 13 completed weeks being 53.5 pg/ml (n=78), 63.1 pg/ml (n=258) and 65.7 pg/ml (n=112) respectively. There was enough control data to look at completed gestational days, and a weighted linear model was found to best fit this (r^2=0.140, p<0.001), (Fig. 2). From this, the raw PP13 MoM was determined for each control and case.

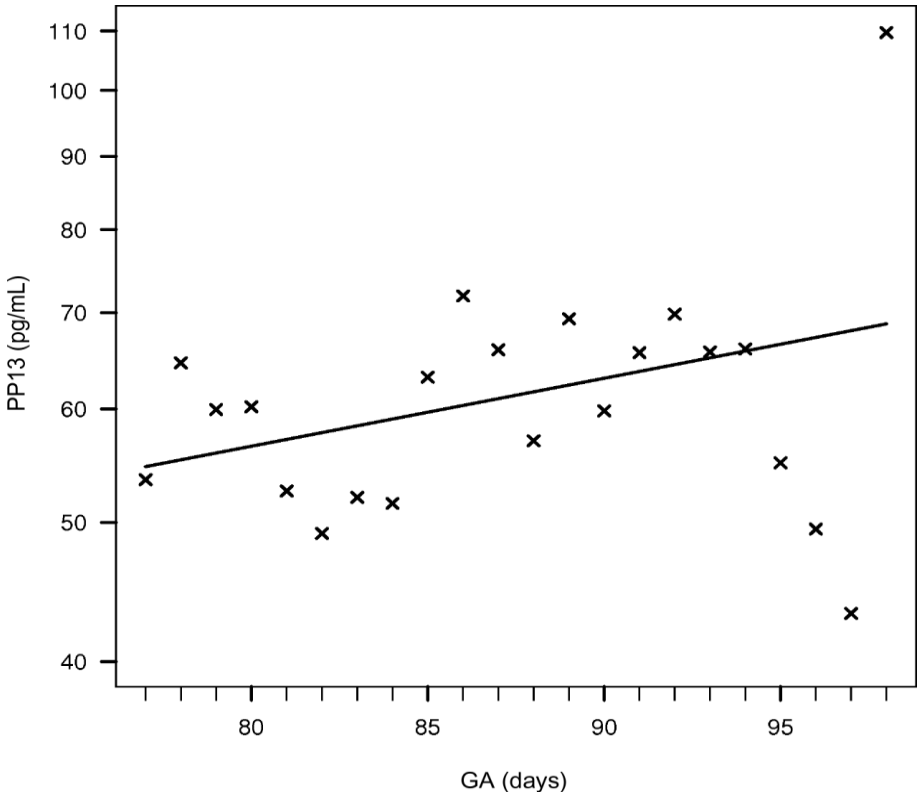

Figure 2. Relationship between PP13 concentration and gestational age. The line shown is a linear regression model: expected PP13 concentration = 5.045 × GA (weeks) - 1.196.

Table 1 Demographic data median variables in controls and each group.

		Screening			Maternal			Fetal & delivery	
	n	GA (days) screening	Maternal age (years)	Smokers (%)	Maternal weight (kg)	Nulliparity (%)	GA (days) delivery	Fetal weight (g)	APGAR
Controls	452	88.00	33.64	10.0	68.00	44.4	279.0	3560.00	9.00
All PE	33	87.00	34.10	12.1	90.60‡	66.7*	263.0‡	2950.00†	8.00‡
All SGA	15	87.00	31.63	13.3	76.45†	60.0	234.0‡	1345.00†	8.00‡
GH	6	88.00	36.16	33.3	86.00†	16.7	277.0	3820.00	8.50
HELLP	6	86.50	34.90	0.0	76.00	33.3	230.5†	1795.00†	8.50*
Early PE	13	87.00	34.10	23.1	83.00‡	46.2	231.0‡	1555.00‡	7.00‡
Late PE	20	87.00	34.01	5.0	94.00‡	80.0†	270.0†	3285.00*	9.00‡
Early SGA	11	88.00	31.75	9.1	75.85*	54.5	224.0‡	1270.00‡	8.00‡
Late SGA	4	79.00*	31.15	25	85.15*	75.0	270.0	2432.50‡	7.50‡
PE and SGA	7	82.00	34.10	14.3	93.00‡	57.1	224.0‡	990.00‡	8.00‡
PE only	21	87.00	33.88	14.3	92.00‡	76.2†	268.0‡	3220.00†	8.00‡
SGA only	8	90.00	31.29*	12.5	69.00	62.5	255.5†	1562.50‡	8.00‡

Symbols denote were a significant difference is found compared to the control group *$p<0.05$, †$p<0.01$, ‡$p<0.001$

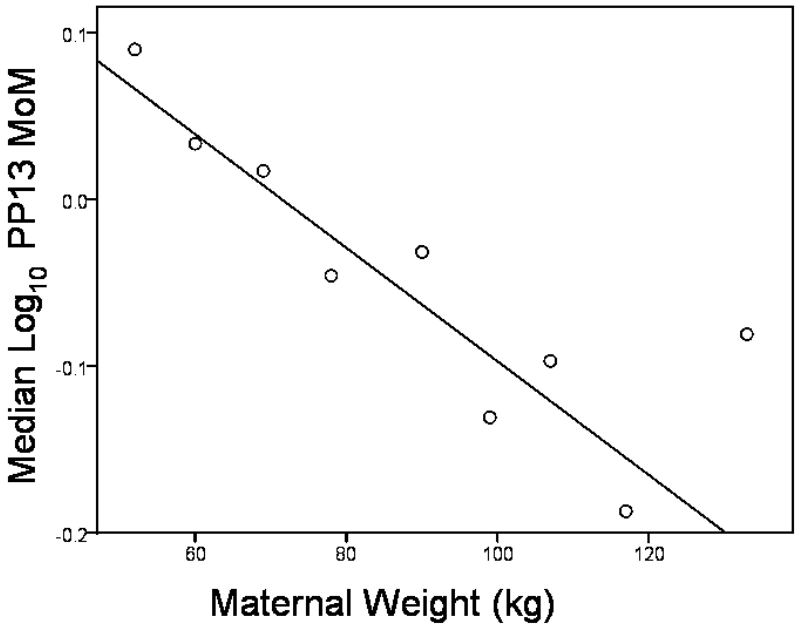

Figure 3. Relationship between log_{10} PP13 MoM and maternal weight. The line shows a log-linear model, weighted by n, with the equation: corrected PP13 MoM = Raw MoM / 10 ^ (-0.003 × Maternal Weight (kg) + 0.243).

Log_{10} PP13 MoMs were found to be related to maternal weight ($p<0.0001$), shown with equation in Figure 3, and case and control pregnancies were corrected for maternal weight using this.

Smokers in the control population were found to have a decreased PP13 median MoM of 0.57 vs non smokers MoM 1.03 ($p<0.0001$). To correct for this, smokers MoMs were divided by 0.57.

Table 2. Summary of PP13 research.

Group	GA (weeks)	n	MoM	p-Value
PE (all[a] or late onset[b])				
Stamatopoulou et al., 2010[a]	11-14	33	1.08	0.61
Stamatopoulou et al., 2010[b]	11-14	20	0.97	0.82
Huppertz et al., 2008[b]	11-15	4	0.15	<0.05
Chafetz et al., 2007[a]	9-12	47	0.20	<0.001
Khalil et al., 2009b[a]	11-14	42	0.40	<0.001
Romero et al., 2008[a]	8-13	50	0.59	<0.001
Spencer et al., 2007b[a]	11-14	88	0.69	<0.001
Spencer et al., 2007b[b]	11-14	44	0.74	<0.001
Akolekar et al, 2009[b]	11-14	160	0.96	N/S
Early onset PE				
Stamatopoulou et al., 2010	11-14	13	1.09	0.59
Nicolaides et al., 2006	11-14	10	0.07	<0.001
Romero et al., 2008	8-13	6	0.26	<0.001
Khalil et al., 2009b	11-14	14	0.30	<0.001
Spencer et al. 2007b	11-14	44	0.63	<0.001
Akolekar et al. 2009	11-14	48	0.83	<0.167
SGA (<5th centile[a], <10th centile[b])				
Stamatopoulou et al., 2010[b]	11-14	15	0.96	0.35
Chafetz et al., 2007[a]	9-12	45	0.60	<0.005
Cowans et al., 2008[b]	11-14	860	1.05	0.93
Cowans et al., 2008[a]	11-14	488	1.06	0.92

MoMs are reported by each study for cases and p-value is from tests for independence (Mann-Whitney-Wilcoxon or Kruskal-Wallis) comparing cases to controls (MoM ≈ 1.0) as reported by each study. N/S=non significant

3.2 PAPP-A

PAPP-A MoMs were correct for gestational age, maternal weight and maternal smoking status at the point of screening.

The corrected median PP13 and PAPP-A MoMs for the control group and each adverse outcome group are shown (Table 3). Significantly lower PAPP-A MoMs were found in the SGA groups both with ($p<0.01$) and without ($p<0.001$) PE. PAPP-A MoMs were also significantly decreased in the all PE group as well as the early PE group ($p<0.05$). We did not find any significant differences between PP13 levels in any group of cases compared to controls.

Table 3. Median MoMs for PP13 and PAPP-A for each group, comparison with controls using Mann-Whitney test. *p<0.05, †p<0.01, ‡p<0.001

	PP13		PAPP-A	
	Median MoM	**p-value**	**Median MoM**	**p-value**
Controls	1.03		0.98	
All PE	1.08	0.61	0.76	<0.01
All SGA	0.96	0.35	0.55	<0.001
GH	0.93	0.49	1.01	0.93
HELLP	0.91	0.35	0.71	<0.05
early PE	1.09	0.59	0.72	<0.01
late PE	0.97	0.82	0.82	0.08
early SGA	0.92	0.29	0.54	<0.001
late SGA	1.12	0.97	0.57	<0.05
PE and SGA	1.09	0.80	0.55	<0.01
PE only	1.21	0.19	0.89	0.20
SGA only	0.91	0.30	0.54	<0.001

The $\log_{10}$ PAPP-A and PP13 MoM are correlated with each other as well as free-β-hCG and ADAM12, but not PlGF (Table 4). Markers free-β-hCG and PAPP-A were measured for fetal anomaly screening purposes, whereas PP13 and ADAM12 were measured in related studies (Matwejew et al., 2010; Cowans et al., 2010).

Table 4. Correlation between $\log_{10}$ PAPP-A and PP13 and other first trimester maternal serum markers.

Markers	**r-Value**	**p-Value**
log PAPP-A vs log fßhCG	0.26	<0.0001
log PAPP-A vs log PlGF	-0.04	0.43
log PAPP-A vs log ADAM12	0.15	<0.001
log PAPP-A vs log PP13	0.13	<0.005
log PP13 vs log fßhCG	0.16	<0.0005
log PP13 vs log PlGF	0.09	0.06
log PP13 vs log ADAM12	0.20	<0.0001

3.3 PlGF

The demographic data for gestational age (weeks), maternal age (years), smoking, parity and maternal weigth (kg) for the controls, early onset and late onset PE, PE±SGA, HELLP Syndrome and GH are shown (Table 1).

In the control group, PlGF concentrations increased with gestational age, and a lweighted regression model was found to best fit this (r2=0.718, p<0.001), (Figure 4), with equation.

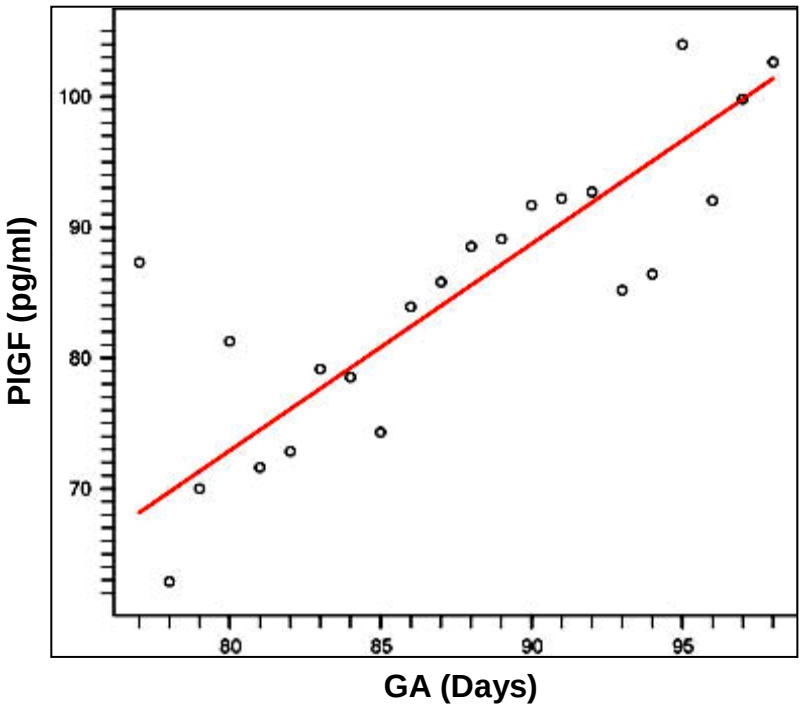

Figure 4. The PlGF concentration changes over gestational age are studied. The dots show actual data points, the line shows a linear regression model weighted by n with the equation: expected PlGF concentration = 1,58 x GA (days) – 53.62.

From this, the raw PlGF MoM was worked out for each control and case PlGF concentration. PlGF MoMs were not found to be related to maternal weight (p=0.574), shown in Figure 5. Smokers in the control population were found to have an increased PlGF median MoM of 1.40 (p<0.0001). To correct for this, smokers MoMs were divided by 1.40.

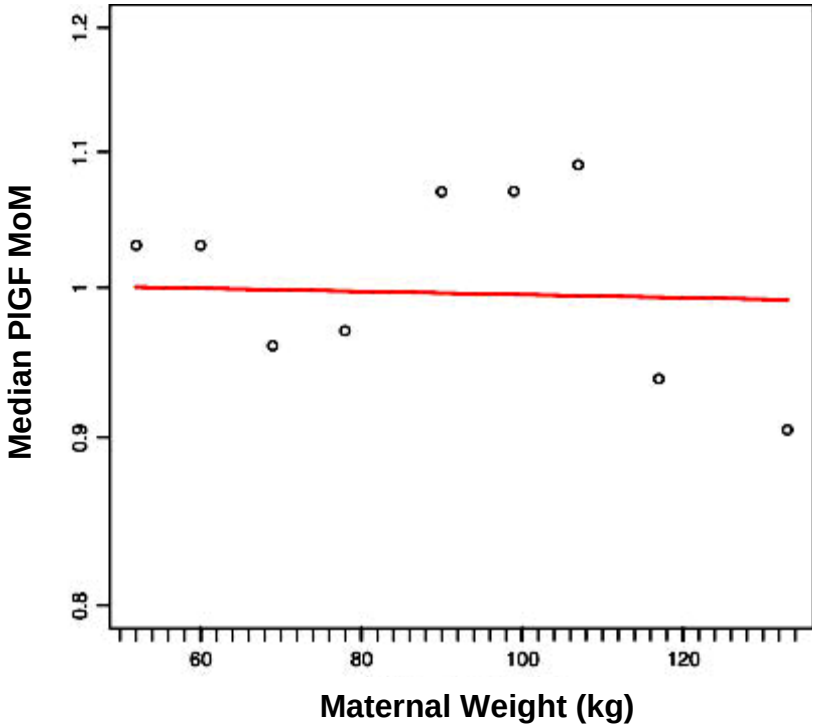

Figure 5. The median PlGF MoM (plotted on log axis) and maternal weight plot. The line shows a regression model, weighted by n.

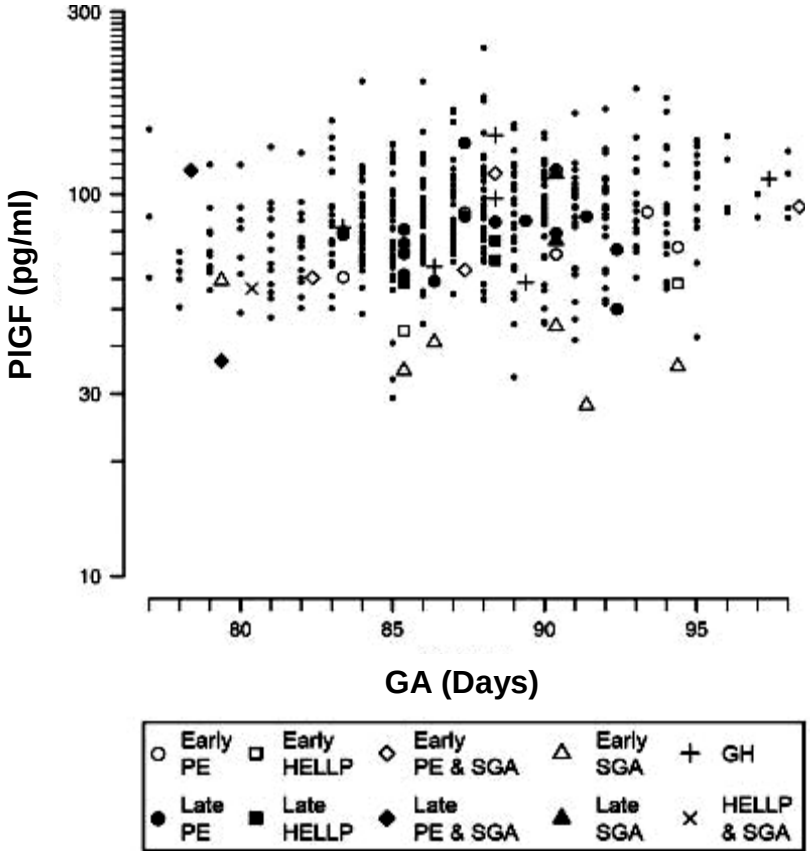

Figure 6. Scatter plots of PlGF concentrations by gestational age at screening.

The corrected median PlGF MoMs for the control group and each adverse outcome group are shown. Significantly lower PlGF MoMs were found in the HELLP, SGA, All PE, Early PE and PE – SGA groups (Table 5).

Table 5. Median MoMs for each group, comparison with controls using Mann-Whitney test.

	N	PlGF median MoM	PlGF IQR	
Controls	452	0.99	0.33	
All PE	33	0.86	0.21	p<0.001
All SGA	15	0.75	0.38	p<0.001
HELLP	6	0.76	0.14	p<0.01
GH	6	0.94	0.30	
PE and SGA	7	0.79	0.17	
PE no SGA/HELLP	21	0.92	0.21	
SGA no PE	8	0.51	0.25	p<0.001
Early PE	13	0.78	0.19	p<0.01
Late PE	20	0.89	0.22	
Early SGA	11	0.75	0.39	p<0.001
Late SGA	4	0.73	0.47	

Bonferroni correction was not used due to its association with reduced statistical power and increase in type II errors.

The log10 PlGF did not correlate with other pregnancy markers measured using the same sample (Table 6). The markers free-β-hCG and PAPP-A were measured for clinical purposes, whereas PP13 and ADAM12 were measured in related studies (Stamatopoulou et al., 2010; Matwejew et al., 2010).

Table 6. Correlation between control group $\log_{10}$ PlGF MoM and other first trimester maternal serum log MoM.

Markers	r value
log PlGF vs. log fßhCG	-0.038
log PAPP-A vs. log PlGF	-0.037
log PP13 vs. log PlGF	0.088
log PlGF vs. log ADAM12	0.014

3.4 ADAM12

The demographic data for gestational age (weeks), maternal age (years), smoking, parity and maternal weigth (kg) for the controls, early onset and late onset PE, PE±SGA, HELLP Syndrome and GH are shown (Table 1).

In one out of 452 controls, one had an undetectable level of ADAM12 and was therefore removed as an outlier.

ADAM12 concentrations increased with gestational age, and a linear model was found to best fit this change ($r^2=0.652$, $p<0.001$), (Figure 6) with equation. From this, the raw ADAM12 MoM was calculated for each ADAM12 concentration.

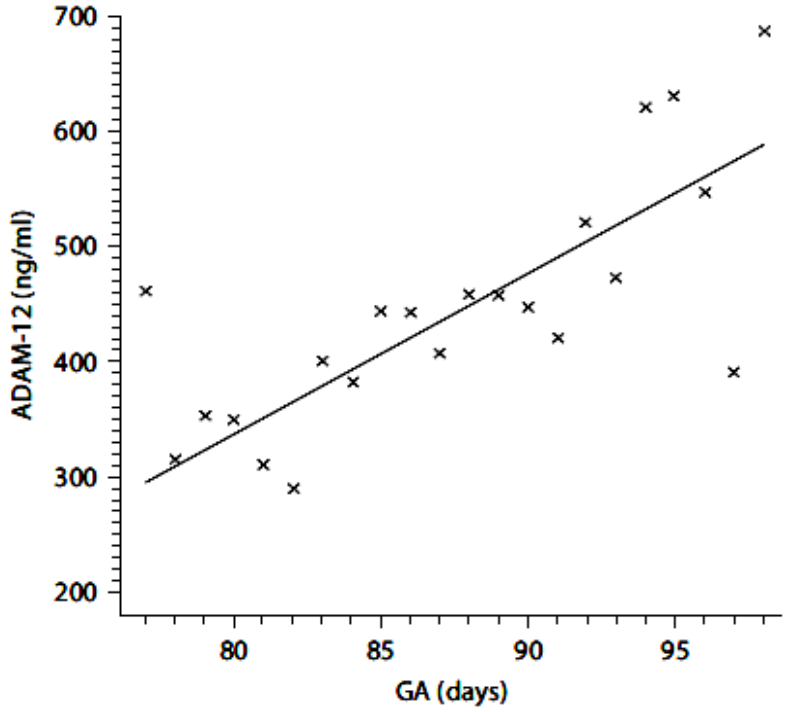

Figure 7. Relationship between ADAM12 concentration and gestational age. The line shown is a linear regression model: expected ADAM12 concentration = 96.1 x gestational age (weeks) - 758.55.

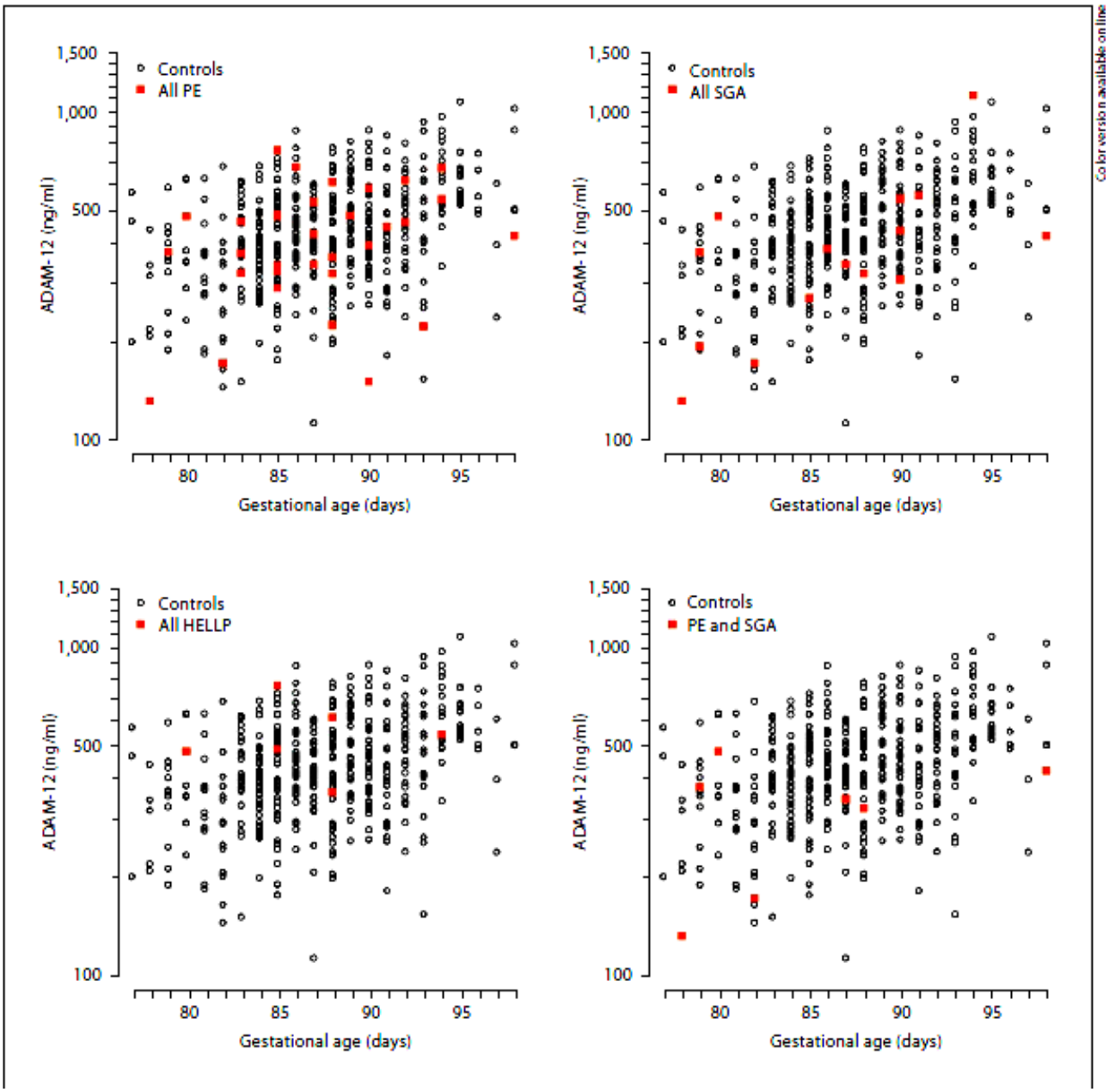

Figure 8. Distribution of ADAM12 values as a function of gestational age in controls and all PE, all SGA, all HELLP and PE & SGA

Raw ADAM12 MoMs were found to be inversely related to maternal weight (r2 = 0.813, p<0.001), (Figure 8), and thus were corrected for using the following log-linear equation:

$$\text{corrected MoM} = \frac{MoM}{10^{-0.0039 \times Weight(kg)+0.2680}}$$

After these corrections, smokers in the control population were found to have a decreased ADAM12 median MoM of 0.88 (p=0.005). To correct for this, smokers maternal weight corrected MoMs were divided by 0.88.

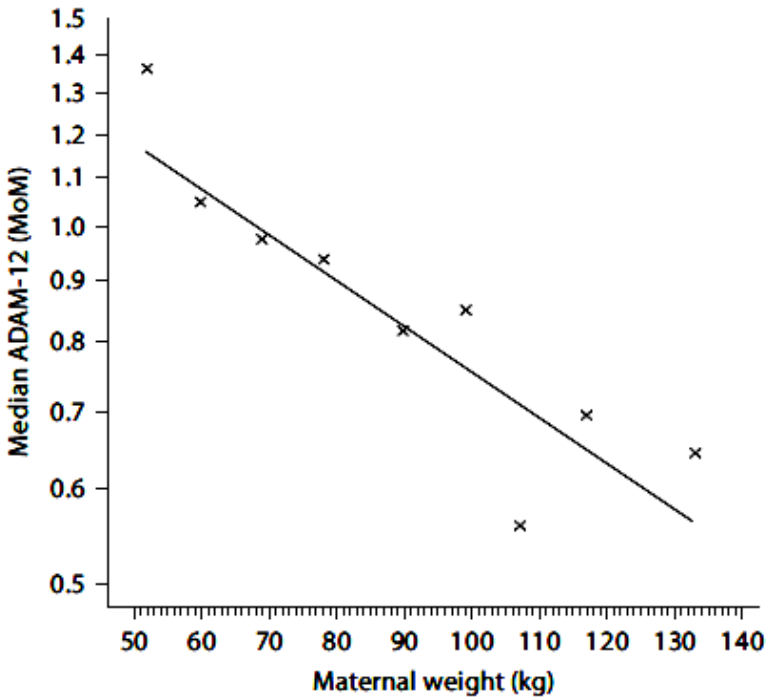

Figure 8. Relationship between $\log_{10}$ ADAM12 concentration and maternal weight. The line shown is a log-linear model: corrected MoM = raw MoM/(10^(–0.0039 x maternal weight (kg) + 0.2680)).

The first trimester median MoM ADAM12 values for control and each adverse outcome pregnancy group are shown (Table 7). Median MoM ADAM12 values were significantly higher than controls in HELLP and late PE group, and were not significantly different to controls in any other group studied.

Table 7. Median raw ADAM12 concentrations (ng/ml), median ADAM12 MoMs corrected for GA and median ADAM12 MoMs corrected for: GA, maternal weight and smoking, for each group.

	Median ADAM12 ng/ml	**Median ADAM12 MoM**	**Median ADAM12 MoM corrected**
Controls	435.9	0.99	1.03 (0.41)
All PE	422.0	0.97	1.16 (0.52)*
All SGA	371.9	0.78	0.91 (0.52)
GH	479.2	1.10	1.20 (0.17)
HELLP	512.0	1.27	1.31 (0.56)*
Early PE	417.0	0.97	1.16 (0.55)
Late PE	432.5	0.94	1.17 (0.45)
Early SGA	416.0	0.90	0.92 (0.40)
Late SGA	249.2*	0.62	0.68 (0.28)
PE and SGA	341.5*	0.71	0.92 (0.71)
PE only	443.1	0.97	1.16 (0.4)*
SGA only	406.7	0.90	0.92 (0.4)

Median MoM comparison with controls using Mann-Whitney test. GA gestational age. *p<0.05.

The correlation between $\log_{10}$ ADAM12 and other pregnancy markers measured using the same sample is shown (Table 8). Other markers were measured either in the clinic, as with free-β-hCG and PAPP-A or in related studies, as with PP13 and PlGF (Cowans et al., 2010; Stamatopoulou et al., 2010). ADAM12 was found to have a significant positive correlation with PAPP-A and PP13.

Table 8. Correlation between log10 ADAM12 and other first trimester maternal serum markers MoMs

Markers	**R value**	
log ADAM12 vs. log fßhCG	-0.012	* p<0.05
log ADAM12 vs. log PAPP-A	0.155‡	† p<0.01
log ADAM12 vs. log PP13	0.203‡	‡ p<0.001
log ADAM12 vs. log PlGF	0.014	

4. Discussion

In nulliparous women the number of total PE, late PE and PE-SGA cases was significantly more prevalent, when compared to control. This general observation supports the view that multiparity is protective of PE, possibly due to previous maternal exposure to the paternal antigen, as previously reported (Roberts et al., 2001) (Table 1).

Increased median maternal weight was also found in all PE groups, as previously reported (Dekker et al., 2001) (Table 1).

Cigarette smoking reduced the levels of PP13 by about half and that of ADAM12, whereas PlGF levels were increased.

This is the first study using the AutoDELFIA (PerkinElmer, Finland) platform to measure PP13, all previous studies were using the manual ELISA (DTL, Israel). We found a small increase in PP13 levels in the control pregnancies over the 11-14 week gestational period studied (Fig. 2), from which we derived a linear model to convert raw PP13 values into MoMs. Previous studies looking at this first trimester gestational age window are not all in agreement with regards to unaffected pregnancy PP13 levels. Some have also found a small positive correlation with gestational age (Chafetz et al., 2007; Romero et al., 2008; Cowans et al., 2008) while others have found no relationship (Huppertz et al., 2008; Nicolaides et al., 2006) and one reported a larger increase from 14.98pg/ml at 11 weeks increasing to 136.64 pg/ml at 13 weeks (Khalil et al., 2009b). Overall, the median levels of PP13 detected using the ELISA in previous studies are roughly 2 fold higher than we found using the

AutoDELFIA, suggesting variation in the two platforms detection of PP13, or platform-to-platform calibration issues. In this study we found it necessary to correct for maternal weight and maternal smoking. Control smokers had a quite drastically reduced levels of PP13, with median MoM almost half that of control non-smokers. Reduction in smokers PP13 levels has been previously reported (Cowans et al., 2007; Khalil et al., 2009b).

The current study does not find any statistically significant relationship between corrected first trimester PP13 levels and the adverse pregnancy outcomes investigated. This is in contrast to the majority of published literature on first trimester PP13, where significantly lower levels of PP13 have been found in PE and SGA cases. Compared to control MoMs of approximately 1, first trimester median PP13 MoMs from women who went on to develop PE have been reported as low as 0.15 ($p<0.05$, n=4) (Huppertz et al., 2008), 0.2 ($p<0.001$, n=47) (Chafetz et al., 2007) 0.4 ($p<0.0001$, n=42) (Khalil et al., 2009b) and 0.59 ($p<0.001$, n=50) (Romero et al., 2008) and for early onset (<34 weeks) PE as low as 0.07 ($p<0.001$, n=10) (Nicolaides et al., 2006), 0.26 ($p<0.001$, n=6) (Romero et al., 2008) and 0.3 ($p<0.0001$, n=14) (Khalil et al., 2009b). In addition, a recent molecular study has found PP13 mRNA expression in 11 week syncytiotrophoblasts of women who developed PE to be lower than in normal pregnancies (Sekizawa et al., 2009). Women who went on to develop SGA have been reported in one study to have lower first trimester PP13 levels compared to controls, with a median MoM of 0.6 ($p<0.005$, n=42) (Chafetz et al., 2007). However, another study found no significant differences in first trimester PP13 levels in pregnancies which went on to deliver SGA (n=860) compared to controls (Cowans et al., 2008).

There are no previous studies looking at first trimester PP13 levels in pregnancies which later develop HELLP, however one group found third trimester PP13 maternal serum levels to be increased in PE and HELLP pregnancies, despite decreased PP13 expression in these tissues, due to an increase in membrane shedding of PP13 (Than et al., 2008).

It is necessary to consider possible reasons for such contrasting results found in this study and a previous study (Cowans et al., 2008) compared to the consensus in the literature. As already mentioned, this study used the new AutoDELFIA platform, which gave median PP13 values for control pregnancies much lower than previously reported. Also this study was performed using samples with a mean storage time of 5 years at -70^{o}C, so stability of PP13 is a possible factor to consider, although controls were matched for storage time and gestation.

Further studies using both the ELISA and AutoDELFIA platforms should be carried out to investigate the level of first trimester PP13 in pregnancies with adverse outcomes. Although the majority of studies suggest PP13 has the potential to be a good marker for PE, especially early onset PE, there is still a huge variation which needs to be addressed before the marker could realistically enter as a marker alone or in combination with other serum or ultrasound markers.

In this study we found decreased levels of first trimester maternal serum PAPP-A in all PE, early PE and SGA (both with and without PE) (Tables 3, 4). This corroborates many previous studies which have found decreases in PAPP-A PE (5,9-12,34) and SGA (Canini et al., 2008; Spencer et al., 2008a, Kang et al., 2008). The low levels of

PAPP-A in these adverse pregnancy outcomes supports PAPP-A functioning as a IGFBP protease in the IGF pathway (Lawrence et al., 1999, Cowans et al., 2007).

This is the first study to use the AutoDELFIA PlGF, rather than the R&D Systems PlGF ELISA platform. PlGF has been found to increase over the gestational window studied, as previously reported (Lambert-Messerlian and Canick, 2004; Akolekar et al., 2008). As found in the study of Akolekar et al (2008) we found a significant increase of PlGF in smokers but unlike this study, we were not confirm a relationship between PlGF and maternal weight nor PlGF and PAPP-A levels (Table 4, 6) (Akolekar et al., 2008). It is unlikely, that the different platforms, AutoDELFIA PlGF vs. the R&D Systems PlGF ELISA are the explanation for these differences.

In pregnancies resulting in SGA, we found PlGF at 11-13^{+6} weeks to be significantly lower compared to control pregnancies (median PlGF MoM: SGA 0.503 vs. control 0.987, $p<0.001$). Several similar studies have looked at PlGF in the first trimester in SGA pregnancies, however, there are inconsistencies in the literature. The first study to look at PlGF levels in first trimester serum of pregnancies that resulted in SGA with birth weight less than 5^{th} centile actually found an increase in cases (median PlGF MoM: SGA 1.57 vs. control 0.98, $p<0.001$) (Ong et al., 2001). Later, a group reported no significant difference in PlGF levels between SGA pregnancies and controls after adjustment for gestational age in serum taken from weeks 7 to 12 (Thadhani et al., 2004). Another study found increased first trimester PlGF levels were associated with a decreased risk of delivering an SGA infant (Smith et al., 2007). A further study, looking at the change in PlGF levels from first to second trimesters found a decrease in raw first trimester PlGF concentrations in 145 SGA pregnancies, not adjusted for gestational age (Erez et al., 2008). A smaller decrease than we found in median

PlGF MoMs has been reported in first trimester serum of SGA pregnancies with birth weight less than 5th centile (median PlGF MoM: SGA 0.900 vs. control 0.991, $p<0.001$) (Poon et al., 2008a). Our data support the idea that fetal growth, and ultimately birth weight, may be related to first trimester placental development.

In this study we also found that at 11 to 13^{+6} weeks, median PlGF MoMs were significantly lower in some hypertensive pregnancy complications studied. There was no significant change in the levels of PlGF in gestational hypertension cases. Similar results have been previously presented (Akolekar et al., 2008). We found PlGF median MoMs to be decreased in HELLP, all PE, early PE and PE-SGA. PlGF has been previously found to be lower during the clinical stages of PE pregnancies (Torry et al., 1998; Reuvekamp et al., 1999). Various studies have also found PlGF levels to be significantly decreased in the second (Tjoa et al., 2001; Su et al., 2001; Taylor et al., 2003; Polliotti et al., 2003; Crispi et al., 2008) and first (Tidwell et al., 2001; Akolekar et al., 2008) trimesters in pregnancies destined for PE. In severe cases of PE, either early onset, or where both PE and SGA occur, PlGF levels are found to be further reduced (Tidwell et al., 2001; Taylor et al., 2003; Polliotti et al., 2003; Crispi et al., 2008). In contrast to these findings, there are a few studies undertaken prior to 20 weeks of gestation where PlGF was found to be unaffected in pregnancies with pre-eclampsia (Ong et al., 2001; Livingston et al., 2001; Lambert-Messerlian and Canick, 2004). PlGF levels have also been found to be decreased in hydatidiform mole pregnancies (Koga et al., 2009) and normotensive but proteinuric pregnancies (Holston et al., 2009).

We were able to detect a significant decrease in PlGF levels in HELLP syndrome, which agrees with previous findings on severe PE. In a previous study of expression of VEGF family members and receptors in situ and in vitro, PlGF was found to be

down regulated in cytotrophoblasts from severe PE and HELLP placental samples (Zhou et al., 2002).

We have shown for PlGF to hold potential usefulness as a first trimester marker for SGA and some forms of PE. However, further studies should be carried out to attempt to reach a consensus on the usefulness of this marker both alone and in combination with ultrasound measurements, on the detection rate for these disorders.

ADAM12 levels were found to steadily increase with gestational age over the window of time studied (Fig. 7). It was also found necessary to account for haemodilution in ADAM12 and to correct for the decreased levels of ADAM12 were found in smokers. All three of these aspects concur with previous studies (Laigaard et al., 2003; Makrydimas et al., 2006; Poon et al., 2008b; Spencer et al., 2008d,e).

In this study we did not find any significant correlation between IUGR, all PE nor early PE first trimester ADAM12 levels and control levels. However, we did find that first trimester levels of ADAM12 are significantly raised in late PE and HELLP compared to control levels. This conflicts previous studies, which have shown levels of ADAM12 to be decreased or not altered at all in pregnancies with hypertensive disorders. In a recent study, Poon et al have shown no significant variation of first trimester ADAM12 levels in a cohort of 128 PE cases and 88 GH cases, and neither was a difference found when comparing early (<34weeks) and late (>34weeks) PE (P=0.871) (Poon et al., 2008). Lower levels of ADAM12 in the serum of PE women was observed in two studies (Laigaard et al., 2005b; Spencer et al., 2008f). In the study by Spencer, when the decreased levels of the molecule were combined with Pulsatility index (PI), there was an increase in the detection rate at 66% during the early first trimester screening.

Another study found increased levels of ADAM12 in the placental tissue and serum of second and third trimester women who developed PE (Gack et al., 2005). Gene expression of ADAM12 at late-second and third trimester showed an upregulation of its transcript among pre-eclamptic tissues. Further analysis of the molecule in maternal serum of the same gestational age, using western blotting, confirm the increase in patients diagnoses with PE. Spencer et al also found marginally increased levels of ADAM12 in the serum of second trimester women developing PE, which is consistent with the data by Gack (Gack et al., 2005; Spencer et al., 2008f). Similarly, the expression of the placental protein PAPP-A is increased in serum and placental tissue during the third trimester in pre-eclamptic pregnancies (Toop and Klopper, 1981; Bersinger et al., 2002, 2003; Gack et al., 2005; Deveci et al., 2009) and has also been shown to be raised in such pregnancies from 22-24 weeks (Spencer et al., 2006). In addition, the upregulation of the PAPP-A gene pappalysin 2 (PAPPA2) has been found in 28 week HELLP pregnancy placentas (Buimer et al., 2008).

ADAM12 has a proteolytic role on insulin like growth factor binding protease-3 (IGFBP-3) (Shi et al., 2000). It has been previously been shown that the levels of IGFBP-3 do not differ in pre-eclamptic and normotesensive women (Varma et al., 1993; Hubinette et al., 2003). These findings support what we have found, in that ADAM12 levels are not altered in all PE and early PE groups.

In this study, first trimester levels of ADAM12 were not found to be significantly different in IUGR cases and controls. This contradicts the findings of other studies, where ADAM12 levels have been found to be decreased in IUGR pregnancies (Cowans and Spencer, 2007; Poon et al., 2008b). ADAM12 is believed to be important in fetal growth, acting to increase levels of bioavailable IGFI and II, through proteolysis of IGFBP-3 (Cowans and Spencer, 2007). Therefore, lower levels of

ADAM12 in early pregnancy were postulated to result in decreased functionality of insulin-like growth factor pathways, leading to IUGR. However, the data found in the current study do not support this.

With regard to the correlation of ADAM12, our data support findings from previous studies where ADAM12 was positively correlated with PAPP-A (Laigaard et al, 2003; Spencer and Cowans, 2007; Poon et al., 2008b). In addition, we have also shown ADAM12 to correlate with PP13.

In this study we have to consider the stability of the samples. This study was performed in samples with a mean storage time of 5 years at -70°C. Although there are few studies on the stability of ADAM12 with time, Laigaard and colleagues excluded analysis of ADAM12 in samples beyond 4 years based on preliminary results they found (Laigaard et al., 2006a). Cowans and Spencer presented data which suggests that there is a significant loss of ADAM12 with time certainly at room temperature over a 3 day period with 50% loss, which confirmed previous findings of Laigaard et al (Laigaard et al., 2003;Cowans and Spencer, 2007). Further studies need to done on the long term stability of ADAM12 at lower storage temperatures.

In conclusion, further studies of cases with PE are required to determine whether ADAM12 has a role to play in the earlier identification of pregnancies destined to develop PE or whether the previous studies showing reduced first trimester levels are merely a reflection of an associated growth restriction. Similarly in cases with HELLP we need more data to establish if the elevation seen in this study has any clinical value.

5. Summary

The rationale for this study was to investigate the potential roles of maternal serum PP13, PAPP-A, PlGF and ADAM12 in the prediction of adverse pregnancy outcomes at 11-13^{+6} weeks gestation. These markers may be useful in establishing a follow up schedule for the pregnancy already at the end of the first trimester, or in the absence of risks to confirm normality. They may also be useful for the identification of pregnancies which may benefit from early Aspirin treatment.

The current study does not find any statistically significant relationship between corrected first trimester PP13 levels and the adverse pregnancy outcomes investigated. This is in contrast to the majority of published literature on first trimester PP13, where significantly lower levels of PP13 have been found in PE and SGA cases.

In this study we found decreased levels of first trimester maternal serum PAPP-A in all PE, early PE and SGA (both with and without PE), which corroborates many previous studies which have found decreases in PAPP-A and PE as well as SGA.
In pregnancies resulting in SGA and in some hypertensive pregnancy complications, such as PE, early PE and PE-SGA as well as HELLP, we found PlGF at 11-13^{+6} weeks to be significantly lower compared to control pregnancies. Our data support the idea that fetal growth, and ultimately birth weight, may be related to first trimester placental development.

The present study did not find any significant correlation between IUGR, all PE nor early PE first trimester ADAM12 levels and control levels. However, we did find that

first trimester levels of ADAM12 are significantly raised in late PE and HELLP compared to control levels.

One limitation of this study is the stability of the samples. This study was performed in samples with a mean storage time of 5 years at -70°C to -20°C. Although this was a case control study with the normal controls matched for gestation and storage time, this has to be considered.

In conclusion this study shows, that maternal serum markers (PP13, PAPP-A, PlGF, ADAM12) at 11-13^{+6} wks of gestation in diseases based on abnormal placentation such as early and late onset PE, PE±SGA, HELLP Syndrome and GH are different from normal and may be useful in the subsequent management of the pregnancy.

6. References

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