Anti-angiogenic factors and pre-eclampsia in type 1 diabetic women

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Abstract

Aims/hypothesis Elevated anti-angiogenic factors such as soluble fms-like tyrosine kinase 1 (sFlt1), a soluble form of vascular endothelial growth factor receptor, and endoglin, a co-receptor for TGF β 1, confer high risk of pre-eclampsia in healthy pregnant women. In this multi-centre prospective study, we determined levels of these

and related factors in pregnant women with type 1 diabetes, a condition associated with a fourfold increase in pre-eclampsia.

Methods Maternal serum sFlt1, endoglin, placental growth factor (PIGF) and pigment epithelial-derived factor were measured in 151 type 1 diabetic and 24 healthy non-diabetic women at each trimester and at term.

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Results Approximately 22% of the diabetic women developed pre-eclampsia, primarily after their third trimester visit. In women with pre-eclampsia (diabetic pre-eclampsia, $n=26$) vs those without hypertensive complications (diabetic normotensive, $n=95$), significant changes in angiogenic factors were observed, predominantly in the early third trimester and prior to clinical manifestation of pre-eclampsia. Serum sFlt1 levels were increased approximately twofold in type 1 diabetic pre-eclampsia vs type 1 diabetic normotensive women at the third trimester visit ($p<0.05$) and the normal rise of PIGF during pregnancy was blunted ($p<0.05$). Among type 1 diabetic women, third trimester sFlt1 and PIGF were inversely related ($r^2=42\%$, $p<0.0001$). Endoglin levels were increased significantly in the diabetic group as a whole vs the non-diabetic group ($p<0.0001$).

Conclusions/interpretation Higher sFlt1 levels, a blunted PIGF rise and an elevated sFlt1/PIGF ratio are predictive of pre-eclampsia in pregnant women with type 1 diabetes. Elevated endoglin levels in women with type 1 diabetes may confer a predisposition to pre-eclampsia and may contribute to the high incidence of pre-eclampsia in this patient group.

Keywords Anti-angiogenic factors · Diabetes · Endoglin · Pigment epithelium-derived factor · Placental growth factor · Pre-eclampsia · Pregnancy · sFlt1 · Soluble VEGF receptors

Abbreviations

CPEP	Calcium for Pre-eclampsia Prevention trial
OUHSC	University of Oklahoma Health Sciences Center
PEDF	pigment epithelium-derived factor
PIGF	placental growth factor
sFlt1	soluble fms-like tyrosine kinase 1
VEGF	vascular endothelial growth factor

Introduction

Pre-eclampsia, a major cause of premature delivery and maternal and fetal death [1], has a much higher incidence in women with type 1 diabetes mellitus than in the non-diabetic population (~20% vs ~5% respectively) [2–5]. The underlying mechanisms for the increased risk of pre-eclampsia in women with diabetes are unknown and predictive measures for its early detection are lacking. Studies of the high incidence of pre-eclampsia in diabetes could yield information pertinent to pre-eclampsia in general and to the mechanisms of more slowly developing vascular complications of diabetes.

Recent evidence indicates that the long-sought ‘circulating factors’ from the placenta which mediate pre-eclampsia may include soluble fms-like tyrosine kinase 1 (sFlt1) (the extracellular fragment of the vascular endothelial growth

factor [VEGF] receptor) and soluble endoglin (a co-receptor for TGF β 1) [6–12], both of which are anti-angiogenic. Specifically, sFlt1 binds to and sequesters pro-angiogenic placental growth factor (PIGF) and VEGF, while endoglin blocks normal TGF β 1 signals [9, 12]. In pre-eclampsia patients, sFlt1 is upregulated in the placenta and elevated in the maternal circulation, while plasma free PIGF and VEGF are decreased [11]. Sera from pre-eclampsia patients inhibit endothelial tube formation (a model of angiogenesis) and also inhibit PIGF- and VEGF-induced renal vasodilatation, effects that can be reproduced by isolated sFlt1 [11]. Increased sFlt1 and reduced PIGF predicted pre-eclampsia in the Calcium for Pre-eclampsia Prevention (CPEP) trial [6]. More recently, endoglin was shown to be elevated in the serum of non-diabetic pre-eclamptic women, correlating with disease severity and falling after delivery [7, 10, 12]. Endoglin impairs binding of TGF β 1 to its receptor, thereby inhibiting endothelial nitric oxide synthase-mediated vasodilatation. Thus, soluble endoglin, in concert with sFlt1, may induce pre-eclampsia [10]. A recent study in non-diabetic women indicated that rising serum endoglin and sFlt1/PIGF ratio herald the onset of pre-eclampsia [7]. Pigment epithelium-derived factor (PEDF), a novel anti-angiogenic protein, is also altered in diabetes and its increased serum levels are associated with diabetic vascular complications [13, 14]. Although it is unclear whether PEDF is released from placental tissues in pre-eclampsia, PEDF could be implicated as a maternal risk factor in the pathogenesis of pre-eclampsia. These previous studies have identified important potential pathogenic mechanisms for pre-eclampsia, but they were conducted in the non-diabetic population and most were not prospective in design. No prospective studies have addressed the high risk of pre-eclampsia in women with pre-gestational type 1 diabetes, a condition associated with alterations of angiogenesis-related factors [15–17].

We recruited a prospective cohort of pregnant women with established type 1 diabetes. Our primary goal was to identify early markers of pre-eclampsia in the context of type 1 diabetes, and hence to explore potential pathogenic mechanisms. For reference, we also recruited a small cohort of healthy non-diabetic pregnant women to define normal values. We aimed for this group to contain a number of participants approximately equal to the expected number of pre-eclampsia cases from the diabetic cohort. Our prospective study did not address pre-eclampsia in the absence of diabetes.

Study participants were from six medical centres in three countries (Norway, Australia and the USA). Serum soluble angiogenesis-related factors (sFlt1, PIGF, endoglin and PEDF) were measured at each trimester to determine their roles as predictors and potential mediators of pre-eclampsia. We hypothesised that alterations in the balance between circulating angiogenic and anti-angiogenic factors would predict pre-eclampsia in type 1 diabetes, would differ from

those described by others in the absence of diabetes and would suggest mechanisms for the increased incidence of pre-eclampsia in type 1 diabetes.

Methods

Participants and specimens

This study was approved by the Institutional Review Boards of all participating institutions and was conducted according to Declaration of Helsinki principles, with written informed consent obtained from all study participants. From 2002 until 2006, 151 pregnant women with documented pre-gestational type 1 diabetes and 24 non-diabetic pregnant women were recruited in the first trimester (~12 weeks) and followed throughout pregnancy. Both diabetic and non-diabetic women were studied at the following centres: the University of Oslo, Norway; the University of Melbourne, Australia; and in the USA at the Medical University of South Carolina; Spartanburg Regional Hospital, SC; the University of Colorado, Denver, CO; and the University of Oklahoma Health Sciences Center (OUHSC), Oklahoma City. Women with the following complications either pre-pregnancy or at the first study visit were excluded: renal impairment (including microalbuminuria), cardiovascular disease, hypertension or other significant medical problems. Clinical data and specimens (plasma, serum and urine) were collected at three study visits (12.2 ± 1.9 , 21.6 ± 1.5 and 31.5 ± 1.7 weeks of gestation [mean $\pm$ SD]; no overlap) and at term (37.6 ± 2.0 weeks). The first three study visits corresponded with late first, mid-second and early third trimesters.

Of the 151 type 1 diabetic women, 13 either experienced early miscarriage (seven women) or were lost to follow-up (six women), so pregnancy was completed and outcome ascertained in 138 women. Of these, 30 women developed pre-eclampsia and 108 were confirmed as being without pre-eclampsia. Thus 22% of the type 1 diabetic women with completed pregnancies and known outcome developed pre-eclampsia, an incidence consistent with previous reports [2–5]. Of the 30 pre-eclampsia cases, 28 were diagnosed after visit 3, while two were diagnosed at or before visit 3. We excluded these two women from analysis, enabling us to consider visit 3 as a ‘pre-pre-eclampsia stage’ for the purpose of identifying predictors of pre-eclampsia. We also excluded three other women who, although originally thought to be free of microalbuminuria (<300 mg/l) at visit 1, were found upon later urine analysis to have had this condition (two developed pre-eclampsia and one did not). Note that these five exclusions did not affect the results/conclusions of any of our statistical analyses. We therefore report on 133 completed pregnancies in type 1 diabetic pregnant women who had normal urinary albumin

excretion at the first visit and had not developed pre-eclampsia at or before their third visit: of these 26 (20%) developed pre-eclampsia.

Of the 24 non-diabetic pregnant women, only one (~4%) developed pre-eclampsia. This woman was excluded from analysis (a prospective investigation of pre-eclampsia in non-diabetic pregnancy was beyond the scope of our study). Also excluded were one woman who miscarried and one who was lost to follow-up. Therefore, data from 21 non-diabetic women were included in the analysis. The cohort of non-diabetic women was included primarily to provide normal ranges for the angiogenic factors; its number was intended to approximate that of the type 1 diabetic pre-eclampsia cases.

Blood was collected after an overnight fast and, in type 1 diabetic women, prior to insulin administration. Serum was prepared by prompt centrifugation (4,000 g) and stored at -80°C . Urine was collected for 24 h, volume recorded and an aliquot stored at -80°C . Samples were accumulated at study centres, then shipped in batches, frozen on dry ice, to OUHSC, where they were again stored at -80°C until the time of laboratory analysis.

Diagnosis of pre-eclampsia and gestational hypertension

Definitions of pre-eclampsia and gestational hypertension were as previously described [1]. Briefly, pre-eclampsia was defined as new-onset hypertension ($>140/90$ mmHg) after 20 weeks of gestation in a previously normotensive woman, accompanied by proteinuria (>300 mg/24 h). If new-onset hypertension without proteinuria was present, then gestational hypertension was diagnosed.

Laboratory measures

HbA_{1c} was measured at each centre by the DCA2000 method (Bayer Diagnostics, Elkhart, IN, USA). Plasma lipids and urine analyses were carried out at the OUHSC Clinical laboratory. Serum free sFlt1, PlGF and endoglin, and plasma PEDF were measured by sandwich ELISA methods according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). The minimum detectable levels were 31.2, 31.2, 125 and 980 pg/ml, respectively. The inter-assay CVs were 8.3%, 4.7%, 4.0% and 9.0%, respectively, and the intra-assay CVs were 2.3%, 3.3%, 2.8% and 5.6%, respectively.

Data analysis

Results were expressed as means $\pm$ SD or SEM as defined. Four groups of pregnancies were considered: diabetic pre-eclampsia (i.e. developed pre-eclampsia), diabetic gestational hypertension (developed gestational hypertension), diabetic normotensive and non-diabetic normotensive. The

latter group was included to provide normal ranges for the angiogenic/anti-angiogenic factors. The diabetic gestational hypertension group was treated separately because of the uncertainty of whether or not gestational hypertension is a precursor to pre-eclampsia.

Our primary aim was to identify risk indicators for pre-eclampsia in type 1 diabetic women. Therefore our primary analysis considered differences at each visit between the diabetic pre-eclampsia and diabetic normotensive groups; these were analysed using unpaired Student's *t* tests or χ^2 tests. All other analyses were secondary and included: (1) differences between the diabetic gestational hypertension group and the other diabetic groups; and (2) differences between the non-diabetic and diabetic normotensive or combined diabetic groups to discern changes in angiogenic

measures that are due to diabetes. The primary time-point of interest was the third visit, reflecting participant status just prior to the diagnosis of pre-eclampsia. Given the limited number of patients who developed pre-eclampsia, an unpaired Student's *t* test was used, instead of a repeated measures analysis approach, to make comparisons between the diabetic groups with and without pre-eclampsia. No adjustments were made to the alpha level for multiple comparisons between groups or across time-points; instead, a hierarchy of questions of primary interest (i.e. the comparisons between diabetic pre-eclampsia and diabetic normotensive groups at visit 3) was specified. Other secondary comparisons were exploratory. The analysis conclusions did not differ when comparisons were made between the diabetic pre-eclampsia vs the combined group

Table 1 Clinical profiles of 133 type 1 diabetic and 21 non-diabetic women during pregnancy

Characteristic	Non-diabetic	Diabetic		<i>p</i> value ^a	
		No pre-eclampsia	Pre-eclampsia		
		No GH	GH		
<i>n</i>	21	95	12	26	
Age (years)	32.0±4.6	30.0±4.9	32.1±3.5	29.2±5.5	0.49
BMI (kg/m ²)	23.5±3.7	25.9±5.2	26.0±3.5	28.0±5.4	0.086
Alcohol use (%)					
None	10	37	25	20	0.46 ^b
None during pregnancy	70	51	67	68	
Smoking (%)					
No	100	81	82	92	0.25 ^b
Stopped in pregnancy	0	8	18	4	
First pregnancy (%)	52	38	27	74	0.0019
Gravida (n)	1.7±1.0	2.1±1.5	1.8±0.6	1.5±1.0	0.028
Para (n)	0.5±0.9	0.5±0.7	0.4±0.5	0.3±0.6	
Abortus (n)	0.1±0.4	0.6±1.1	0.5±0.5	0.2±0.5	
Age at diabetes onset (years)	—	17±8	16±8	12±7	0.0072
Duration of diabetes (years)	—	13±8	15±7	16±7	0.026
HbA _{1c} (%)	5.3±0.3	6.8±1.1	7.0±1.7	7.3±1.1	0.14
Blood pressure (mmHg)					
Systolic	112±9	112±12	113±9	115±13	0.42
Diastolic	67±8	67±8	69±6	67±9	0.94
Microalbumin (mg/l)	4.5±1.7	12.4±35.0	4.7±2.0	10.3±17.8	0.7
Total cholesterol (mmol/l)	4.84±0.65	4.58±0.73	4.27±0.36	4.71±0.70	0.39
HDL-cholesterol (mmol/l)	2.12±0.57	2.07±0.47	1.99±0.23	1.92±0.36	0.11
LDL-cholesterol (mmol/l)	2.25±0.75	2.10±0.65	1.94±0.41	2.33±0.70	0.15
Triacylglycerol (mmol/l)	1.04±0.38	0.93±0.41	0.77±0.16	1.04±0.41	0.22
Gestational age (week)					
Visit 1	12.7±1.7	12.1±1.8	11.7±1.8	12.2±2.0	0.73
Visit 2	21.5±1.2	21.5±1.4	20.8±1.4	21.9±1.7	0.28
Visit 3	31.2±1.1	31.5±1.7	32.5±1.9	31.7±1.7	0.62
Term	39.2±1.5	37.5±2.0	37.4±1.3	37.1±1.3	0.27

Values are means±SD; measurements refer to visit 1 unless otherwise indicated

GH, gestational hypertension

^a Diabetic normotensive vs diabetic pre-eclampsia

^b *p* value refers to combined percentage, i.e. None and None during pregnancy or No and Stopped in pregnancy

of diabetic gestational hypertension and diabetic normotensive participants (instead of vs diabetic normotensive alone).

Analyses of the ratio sFlt1/PlGF and relationship between sFlt1 and PlGF were conducted after logarithmic transformation. All tests were two-tailed, with $p < 0.05$ considered significant. Multiple regression for pre-eclampsia to determine joint effects of the angiogenic factors used a logistic regression with stepwise selection of significant predictors with a significance level of 0.05 to enter the analysis and a level of 0.10 to be removed from the analysis. All statistical analyses used SAS (version 9.1; SAS Institute, Cary, NC, USA).

Results

Clinical characteristics at visit 1

Table 1 shows the clinical characteristics of 133 type 1 diabetic and 21 non-diabetic women at visit 1. Most (95%) participants were white. Type 1 diabetic women were categorised according to the subsequent presence or absence of pre-eclampsia or gestational hypertension, with 26 (20%) developing pre-eclampsia, and 12 (9%) developing gestational hypertension but not pre-eclampsia. The 21 non-diabetic women provided values for normal pregnancy. No differences were observed across groups with regard to age, blood pressure, smoking or alcohol use. There was a significantly higher percentage of first pregnancies among type 1 diabetic women who developed pre-eclampsia (diabetic pre-eclampsia, 74%) than among those who were free of hypertensive complications (diabetic normotensive, 38%) ($p < 0.01$). Diabetic pre-eclampsia women had a younger age of diabetes onset ($p < 0.01$) and longer diabetes duration ($p < 0.05$) than diabetic normotensive women. As expected, HbA_{1c} was higher in diabetic than in non-diabetic women, but did not differ among the three diabetic groups. Measures of conventional lipids showed no differences between the diabetic pre-eclampsia and diabetic normotensive women.

Longitudinal changes of weight, HbA_{1c}, mean arterial pressure and urine microalbumin/creatinine ratio

We first investigated alterations of risk markers including body weight, HbA_{1c}, mean arterial pressure and urine microalbumin/creatinine ratio throughout gestation in four groups of women (Fig. 1). Weight increased gradually in all groups during pregnancy. However, the diabetic women who developed pre-eclampsia were heavier at all visits than those who did not, although this only reached significance at term ($p < 0.05$; Fig. 1a). Levels of HbA_{1c} dropped slightly at visit 2, but were not significantly different among the three diabetic groups (Fig. 1b). Mean arterial pressure was

Fig. 1 Longitudinal changes of (a) gestational weight, (b) HbA_{1c}, (c) mean arterial blood pressure (MAP) and (d) urine microalbumin/creatinine ratio during gestation. Values (means±SEM) were plotted against the average gestational age at each visit and at term. For illustrative purposes, four groups of participants are shown. White square, dashed line, non-diabetic normotensive; white circle, diabetic normotensive; grey circle, diabetic gestational hypertension; black circle, diabetic pre-eclampsia. Values significantly different between participant groups ($p < 0.05$) are indicated: the primary analysis compared diabetic pre-eclampsia and diabetic normotensive groups ($*p < 0.05$). Secondary analyses compared between diabetic gestational hypertension vs diabetic normotensive groups ($†p < 0.05$) and diabetic normotensive vs non-diabetic normotensive groups ($‡p < 0.05$). Additionally, the following values reached statistical significance ($p < 0.05$) compared with visit 1 (baseline): a gestational weight in all four participant groups at visit 3 and term; b HbA_{1c} in diabetic normotensive and diabetic pre-eclampsia groups at visits 2, 3 and term, and non-diabetic normotensive subjects at visit 2; c mean arterial pressure in diabetic pre-eclampsia participants at both visit 3 and term, and diabetic normotensive and diabetic gestational hypertension participants at term

significantly increased at visits 2 and 3 in the diabetic women who developed pre-eclampsia compared with those who remained normotensive (Fig. 1c). Urine microalbumin/creatinine ratio was stable during the first three visits and by definition was greatly increased at term in diabetic women with subsequent pre-eclampsia, whereas lesser increases were seen in the other two diabetic groups (Fig. 1d).

Angiogenic/anti-angiogenic factors

sFlt1 In non-diabetic women, serum sFlt1 (Fig. 2a) was stable from visit 1 to visit 3, increasing at term. In type 1 diabetic women, sFlt1 increased progressively after visit 2. This increase was most pronounced in the diabetic women with pre-eclampsia, reaching approximately twice the level of the diabetic normotensive women at visit 3 ($p<0.05$) and remaining 20% higher at term ($p<0.05$).

PlGF As seen in Fig. 2b, serum PlGF increased progressively in non-diabetic normotensive women from visit 1 to visit 3, declining at term. In type 1 diabetic women the increase at visit 3 was blunted, being most marked in

women who subsequently developed pre-eclampsia (diabetic pre-eclampsia vs diabetic normotensive, $p<0.05$).

Endoglin In non-diabetic women, soluble endoglin levels (Fig. 2c) increased slightly at visit 3 and more markedly at term. In women with type 1 diabetes, the increases at visit 3 were significantly more pronounced ($p<0.0001$ vs non-diabetic group), but were of a similar magnitude in all three diabetic groups.

PEDF In non-diabetic women, PEDF increased steadily with gestation (Fig. 2d). Levels were generally higher in the presence of diabetes, with type 1 diabetic pre-eclampsia women having higher levels than diabetic normotensive women at visit 2 ($p=0.06$). At visit 3, as with endoglin, levels were elevated in the combined diabetic groups compared with non-diabetic women ($p=0.04$).

Fig. 2 Longitudinal changes of serum angiogenic/anti-angiogenic factors: **a** sFlt1, **b** PlGF, **c** endoglin and **d** PEDF during gestation. See Fig. 1 for keys for both primary and secondary analyses. Additionally, the following values reached statistical significance ($p<0.05$) compared with visit 1 (baseline): **a** sFlt1 and **c** endoglin in all three diabetic groups (normotensive, gestational hypertension and pre-eclampsia) at visit 3 and term, and non-diabetic normotensive subjects at term; **b** PlGF in diabetic normotensive and diabetic pre-eclampsia subjects at visits 2 and 3, and non-diabetic normotensive group at visits 2, 3 and term; **d** PEDF in diabetic normotensive and diabetic pre-eclampsia subjects, and non-diabetic normotensive group at visit 3 and term

Relationship between sFlt1 and PlGF

Since sFlt1 may promote pre-eclampsia by neutralising PlGF, we explored the relationship between these two factors. The sFlt1/PlGF ratio throughout pregnancy is shown in Fig. 3a. With and without logarithmic transformation, the ratio was increased significantly at visit 3 in diabetic pre-eclampsia vs diabetic normotensive women. The inverse relation between sFlt1 and PlGF at visit 3, which is linear after logarithmic transformation, is shown in Fig. 3b. This inverse correlation was significant in the diabetic group ($r^2=42\%$, $p<0.0001$) only at visit 3.

Fig. 3 Relationship between serum levels of sFlt1 and PlGF during gestation. See Fig. 1 for keys. **a** Changes of the ratio of serum sFlt1/PlGF (logarithmic scale) throughout gestation. In addition, the following values were significantly different ($p<0.05$) from visit 1 (baseline): all four subject groups at visit 2, non-diabetic and diabetic normotensive groups at visit 3, and all three diabetic groups at term. **b** Regression analysis of sFlt1 and PlGF on logarithmic scale at visit 3. For all diabetic women at visit 3, $r^2=42\%$, $p<0.0001$ ($r^2=11\%$ for non-diabetic women). This inverse correlation was not observed at either visit 1 or visit 2, indicating the biological interactions of sFlt1 and PlGF occur at visit 3, immediately prior to clinical development of pre-eclampsia

Joint effects of angiogenic factors

Risk of pre-eclampsia in the presence of type 1 diabetes was modelled in diabetic pre-eclampsia and diabetic normotensive women using logistic regression; the other two groups were excluded from this analysis. Since no significant effects were seen between diabetic pre-eclampsia and diabetic normotensive women at visits 1 and 2, only visit 3 measures of angiogenesis-related factors were considered. These factors were: sFlt1, PlGF, sFlt1/PlGF, endoglin and PEDF. The most parsimonious model included just sFlt1 ($p=0.0005$ to include) with no significant lack-of-fit remaining (Hosmer and Lemeshow Goodness-of-fit residual χ^2 , $p=0.12$). Endoglin showed a marginal effect with $p=0.05$ to include.

Discussion

The most important finding from this study is that alterations of angiogenic and anti-angiogenic factors occur prior to the clinical manifestation of pre-eclampsia in type 1 diabetic pregnancy, and hence, as in non-diabetic women, could play a role in the development of pre-eclampsia. Some of the findings parallel those in non-diabetic women [6]: thus elevated sFlt1, low PlGF and elevated ratio of sFlt1/PlGF are predictive of pre-eclampsia and may be mechanistically implicated. In contrast, elevated endoglin levels do not predict pre-eclampsia in women with type 1 diabetes as they do in non-diabetic women, but, interestingly, this is because endoglin is always elevated in association with late pregnancy in type 1 diabetes. This elevation of endoglin in pregnant type 1 diabetic women could explain, at least in part, the high incidence of pre-eclampsia in this patient group. If pre-eclampsia is dependent upon elevation of both sFlt1 and endoglin, women with type 1 diabetes are clearly predisposed.

Our results show that increased serum levels of sFlt1 and blunted responses of PlGF are present early in the third trimester and precede pre-eclampsia in women with type 1 diabetes, consistent with the findings in a recent, retrospective study of non-diabetic women from the CPEP trial [6]. sFlt1 binds to and neutralises PlGF so that the latter cannot reach its receptor [9, 11]; our data are consistent with this mechanism of action in that serum sFlt1 levels were significantly and inversely correlated with PlGF levels at the third trimester in type 1 diabetic patients. No apparent inverse correlation was observed at either the first or second trimester, suggesting that interactions of sFlt1 and PlGF occur early in the third trimester, but prior to the clinical diagnosis of pre-eclampsia.

Recent studies have shown that soluble endoglin is elevated in the serum of non-diabetic pre-eclamptic women,

preceding the clinical signs of pre-eclampsia [7, 10, 12]. Increased levels of both soluble endoglin and sFlt1 appear to be required for pre-eclampsia to develop. Our data show that, in all three diabetic groups, endoglin levels were significantly increased at visit 3 compared with non-diabetic women, but were unaltered in the early stages of gestation. However, at visit 3, no significant difference in endoglin levels was found between diabetic women with and those without pre-eclampsia (whether the latter group is normotensive, gestational hypertension or both combined). These results indicate that, in contrast to non-diabetic populations, endoglin cannot be used as an early marker to predict pre-eclampsia in type 1 diabetic pregnancy. Diabetes as such did not affect the basal concentration of endoglin; rather, the effect of diabetes was only evident late in pregnancy, at visit 3, and at term. There may be a ‘ceiling effect’ for endoglin which limits a further increase in diabetic women who develop pre-eclampsia. The increase in endoglin in late pregnancy in diabetes could contribute to the increased incidence of pre-eclampsia by providing a ‘fertile soil’, i.e. only elevation of sFlt1 is needed for pre-eclampsia to develop in diabetic patients. To our knowledge, this is the first report to show that serum endoglin levels are elevated in pregnancies complicated by diabetes. Since endoglin impairs binding of TGF β 1 to its receptor, inhibiting not only growth signals but also vascular contractility, the present discovery implies that increased systemic endoglin levels could mediate the development of diabetic vascular complications.

PEDF, which has anti-oxidant, anti-inflammatory and anti-angiogenic effects, has been closely associated with diabetic vascular disease, particularly retinopathy [13, 14]. Plasma PEDF levels are increased in metabolic syndrome [18] and in type 1 and type 2 diabetes in the presence of complications [19, 20]. In our own cross-sectional study of type 1 diabetic patients, serum PEDF levels were positively related to renal dysfunction and pulse pressure (which reflects stiffer arteries) and inversely to small artery elasticity [19]. These findings prompted us to assess the blood levels of PEDF in our study cohort. The results showed that, at visit 1, PEDF was slightly higher in type 1 diabetic than in non-diabetic women. At visit 2, PEDF was higher in diabetic women with than in those without subsequent pre-eclampsia ($p=0.06$), but in the later stages of pregnancy this difference was less marked. However, at visit 3, diabetic women exhibited a general increase in PEDF levels compared with non-diabetic women, suggesting that, as with endoglin, elevation of anti-angiogenic PEDF in diabetes might contribute to a ‘fertile soil’ for pre-eclampsia development. Whether changes in systemic PEDF levels represent compensatory or pathogenic mechanisms is unknown.

We noted a significantly higher proportion of first pregnancies in the type 1 diabetic women with than in those without pre-eclampsia ($p<0.01$; Table 1). This

observation agrees with the previous epidemiological evidence that nulliparity is a predominant independent risk factor for pre-eclampsia [21–23] and that (non-diabetic) women with a first pregnancy have a three- to fivefold increased risk compared with subsequent pregnancies [24]. There is evidence that serum sFlt1 levels are significantly increased in the first compared with second pregnancies, which may account in part for the increased risk of pre-eclampsia among nulliparous women [25]. Interestingly, our own results also indicated a consistent trend towards higher sFlt1 levels in the first than in subsequent pregnancies both in diabetic and non-diabetic groups (data not shown), but this did not reach statistical significance.

Epidemiological evidence indicates that smoking protects against pre-eclampsia, perhaps by promoting angiogenesis [7, 26]. A plausible mechanism was recently revealed: smoking may reduce sFlt1 levels, which could explain its pro-angiogenic effects and its protective effects against pre-eclampsia [27]. However, we did not find significant differences in the proportion of smokers across the three diabetic groups.

In our secondary analyses, levels of angiogenic/anti-angiogenic factors were also explored in type 1 diabetic women with gestational hypertension. Similar to women with pre-eclampsia, type 1 diabetic women with gestational hypertension exhibited blunted PIGF levels at visit 3. Endoglin levels were also significantly elevated at visit 3 compared with healthy women, but were similar to the other diabetic groups. Levels of sFlt1 and PEDF were intermediate between those of diabetic normotensive women and those of diabetic women with pre-eclampsia, and were not statistically different from the diabetic normotensive women. Analyses are limited by the small number of women in this group. The data are consistent with gestational hypertension representing an atypical, mild or incomplete form of pre-eclampsia.

In conclusion, to our knowledge, this is the first investigation to assess angiogenic and anti-angiogenic factors in a multi-centre, prospective cohort of pregnant women with established type 1 diabetes, a condition that confers a greatly increased risk of pre-eclampsia. The results indicate that increased serum level of sFlt1, reduced PIGF level and elevated ratio of sFlt1/PIGF in the third trimester are potential predictors of pre-eclampsia in type 1 diabetic pregnancies. Endoglin does not predict pre-eclampsia in type 1 diabetic women, but its increased levels in the third trimester in pregnancies complicated by type 1 diabetes, regardless of subsequent pre-eclampsia status, could predispose such women to developing pre-eclampsia. Similar considerations apply to PEDF. Due to the limited size of our cohort, especially that of our non-diabetic group, and the absence of non-diabetic participants with pre-eclampsia (precluded because of the large number required for a prospective study given the 4% case yield), these findings need to be confirmed

in larger diabetic and non-diabetic pregnancy cohorts. Future studies should also address the role of angiogenic/anti-angiogenic factors in pre-eclampsia in pregnancies complicated by type 2 and gestational diabetes, and should also pursue mechanisms and preventive measures.

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