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Alterations in fibrin formation and fibrinolysis in early onset-preeclampsia: Association with disease severity

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ABSTRACT

Objective: ; Early-onset preeclampsia is a rare pregnancy-specific disorder associated with significantly increased maternal and fetal morbidity and mortality. Whilst it is known that even normotensive pregnancies are associated with changes in clot formation and dissolution, the nature of how these changes differ in those with early onset preeclampsia has not been well established.

We sought to evaluate parameters of fibrin formation and fibrinolysis in individuals with early onset preeclampsia in comparison to both pregnant and non-pregnant controls. Furthermore, such parameters were correlated with markers of disease severity in this patient cohort, including the presence of multiorgan involvement, the rate of disease progression and the extent of the anti-angiogenic state in this condition.

Study design: ; Patients with early onset preeclampsia (N = 20) and both pregnant (N = 16) and non -pregnant (N = 16) controls were recruited from the cohort at a large urban maternity hospital which saw over 15,000 deliveries during the study period. Platelet poor plasma was prepared from collected whole blood and analysed for parameters of fibrin formation and fibrinolysis (lagtime to and rate of fibrin formation; PAI-1; PAI-2; D-dimer; plasmin-antiplasmin; tPA) in addition to markers of angiogenesis (sFLT-1; Endoglin) using commercially available specific immunoassays.

Results: ; The maximum rate of fibrin formation as well as PAI-1, PAI-2 and D-dimer levels were all significantly increased in those with early onset preeclampsia and pregnant controls when compared to non-pregnant controls without significant differences between the 2 former groups. Plasmin-antiplasmin levels were significantly reduced in a similar manner. tPA levels were significantly elevated in EOP compared to both pregnant and non-pregnant controls. EOP was associated with significantly increased anti-angiogenic factors (sFLT-1; Endoglin) when compared to both pregnant and non-pregnant controls.

Conclusion: ; Markers of fibrin formation and fibrinolysis are significantly alerted in early onset preeclampsia; furthermore, certain markers correlate with disease severity in this patient cohort.

Abbreviations: EOP, Early Onset Preeclampsia; PAI-1, Plasminogen activator inhibitor 1; PAI-2, Plasminogen activator inhibitor 2; PAP, plasmin-antiplasmin; PC, pregnant controls; PLGF, Placental growth factor; sFLT-1, soluble fm-like tyrsosine kinase 1; tPA, Tissue Plasminogen activator.

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Introduction

Preeclampsia is a pregnancy-specific multisystem disorder characterised by new onset hypertension in the setting of significant proteinuria; or new onset hypertension with end organ damage with or without proteinuria, occurring after 20 weeks' gestation [1]. It complicates up to 4 million pregnancies annually worldwide accounting for in excess of 50,000 maternal deaths as well as being associated with significantly increased fetal morbidity and mortality [2,3].

Preeclampsia occurs through a combination of maternal, fetal and placental factors [4]. Whilst the pathogenesis is not fully understood, failure of adequate spiral artery maturation and shallow placentation resulting in altered placental villous maturation has been described [5]. Secretion of placental antiangiogenic factors in turn causes widespread maternal endothelial dysfunction leading to characteristic clinical manifestations [6]. Preeclampsia occurring before 34 weeks' gestation is defined as early onset preeclampsia (EOP) [7]. It exhibits a severe disease phenotype and is characterised by increased placental dysfunction and maternal inflammation compared to late onset preeclampsia (LOP; >34 weeks' gestation). It is as such associated with poorer maternal and fetal outcomes [8,9].

Healthy pregnancy results in significant coagulation and fibrinolysis pathway alterations which result in a prothrombotic tendency; a necessary alteration to reduce post-partum haemorrhage [4,6,10]. The coagulation-fibrinolysis pathway has been shown to be further deranged in preeclampsia with significant hypercoagulability evident; fibrin deposition at the glomerulus is well described in preeclampsia whilst severe disease has been associated with widespread fibrin deposition [10,11].

The primary aim of this study was to assess and compare parameters of fibrin formation and fibrinolysis in non-pregnant controls, pregnant controls, and patients with early-onset preeclampsia. Its secondary aim was to determine whether alterations in these parameters are associated with severity of disease in early-onset preeclampsia. Severity was assessed according to evidence of multiorgan involvement, rate of disease progression, and levels of anti-angiogenesis factors as a correlate for the severity of anti-angiogenic state.

Methods

Patient recruitment

This study was performed in the Rotunda Hospital, Dublin, Ireland, a tertiary referral centre with almost 9000 deliveries per year. Ethical approval was obtained from the Hospital Research Ethics Committee. Pregnant women with EOP, age and gestation-matched healthy pregnant controls (PC) and healthy non-pregnant controls (NPC) were recruited by a senior obstetric fellow with specific expertise in phlebotomy. In accordance with international clinical guidelines [1,7,12], EOP was defined as preeclampsia (new hypertension after 20 weeks gestation with significant proteinuria) developing before 34 completed gestational weeks. Patients with severe preeclampsia were characterised by any of the following: severe hypertension (SBP > 160 or DBP>110 mmHg) twice ≥4 h apart, thrombocytopenia (platelet count <100,000/μl), impaired hepatic function or severe unexplained right upper quadrant abdominal pain, new-onset renal impairment, pulmonary oedema or new-onset cerebral or visual disturbances. Patients with severe anaemia and patients receiving prophylactic or therapeutic anticoagulant therapy or with known previous thrombosis or inflammatory disorders (eg systemic lupus erythematosus) were excluded. Patients were recruited over a 21-month period, during which 15,299 deliveries were recorded in women with pregnancies of gestation >24 weeks. Of these, 334 (2.2%) were complicated by preeclampsia. The majority (294) were classified as late-onset (>34 weeks gestation) while 40 were classified as EOP (<34 weeks gestation). Recruitment was not possible in 2 cases due to brevity of the interval between clinical presentation and delivery. Therefore recruitment was considered in 38 patients. Of these, 10 were excluded due to established exclusion criteria. Of the remaining 28, 26 provided consent for recruitment. 14 and 12 patients with EOP had features of non-severe and severe disease at enrolment respectively. Of these, plasma samples were available for 20 EOP patients (11 with non-severe and 9 with severe disease). 16 NPC and 17 PC were also recruited. Differences in baseline characteristics are detailed in Table 1.

Blood collection and preparation

This study was completed in line with the Declaration of Helsinki. Voluntary, informed, written consent was obtained all participants. 10 ml blood samples were collected into vacutainers containing sodium citrate (final concentration; 0.106 nM). Plasma was prepared by centrifugation at 3000 g for 20 min. Aliquots were frozen at −80 °C until analysis.

Turbidity assay

Turbidmetric analysis of plasma clots was performed essentially as previously described. In brief, 25 μl of plasma was added to the well of a low binding polystyrene 96 well plate. To initiate fibrin clot formation 75 μl of a reaction mixture containing 0.6 pM human tissue factor (Innovin), 10μM phospholipid vesicles and

Table 1
Patient demographic information (mean ± standard deviation).

	Non-pregnant controls	Pregnant controls	Early-onset preeclampsia	p-value*
n	16	16	20	–
Age (years)	32.8 ± 6.3	32.8 ± 5	36 ± 5.3	0.15
BMI (kg/m^2)	24.4 ± 3.7	25.3 ± 5.4	28.6 ± 5.1	0.008
****Gestation (weeks)**	n/a	30.2 ± 2.9	32.1 ± 1.8	0.02
Lagtime to fibrin formation (secs)	320.4 ± 117.3	285.9 ± 46	313.9 ± 88.1	0.4
Vmax of fibrin formation	42.7 ± 16.6	76.5 ± 23.7	65.2 ± 25.6	0.0002
tPA (pg/ml)	1703 ± 788	1767 ± 734	6919 ± 3116	<0.0001
PAI-1 (ng/ml)	22.1 ± 1.6	63.2 ± 24.4	80.6 ± 28.6	<0.0001
PAI-2 (ng/ml)	28.6 ± 68.5	168.7 ± 66.9	122.1 ± 64.2	<0.0001
PAI-1:PAI-2	4.8 ± 3.8	0.4 ± 0.2	0.9 ± 0.6	<0.0001
Plasmin-antiplasmin (ng/ml)	551.9 ± 172.6	381.1 ± 97.4	423.5 ± 114.9	<0.0001
D-Dimer (μg/ml)	0.64 ± 0.44	3.9 ± 1.9	4.47 ± 2.3	<0.0001
sFLT-1 (pg/ml)	140.4 ± 227.1	2880 ± 2606	13788 ± 4328	<0.0001
Endoglin (pg/ml)	3.4 ± 0.9	6.7 ± 3.8	33.5 ± 10.6	<0.0001

*One-way ANOVA (parametric data sets) or Kruskal Wallis test (non-parametric data sets).
**Student *t*-test.

10 mM $CaCl_2$ diluted in 20 mM HEPES, 122 mM NaCl, pH 7.4 was added to each well. Concentrations stated are the final concentration in the 100 μl reaction. Turbidity was measured at 405 nm every min for 120 min. Each sample was analysed in triplicate.

Fibrinolysis markers

Plasma plasminogen activator inhibitor-1 (PAI-1; Diagnostica Stago UK Ltd, Theale, UK), PAI-2 (Invitech Ltd., Huntingdon, UK), tissue plasminogen activator (tPA; Biotechne, Abingdon, UK), and plasmin antiplasmin (PAP; Immunodiagnostic Systems (IDS) Ltd, Tyne&Wear, UK) levels were measured by ELISA according to the manufacturer's instructions. The intra-assay and inter-assay variable for PAI-1 were 3.3% and 2.9%, for PAI-2 were 4.2% and 7.3%, for tPA were 5% and 4% and for PAP were 4.2% and 7.3%, respectively.

Angiogenesis markers

Plasma soluble fms-like tyrosine kinase 1 (sFLT-1), placental growth factor (PLGF), and endoglin levels were measured by ELISA (Biotechne, Abingdon, UK), according to the manufacturer's instructions.

Statistics

Data were analysed using GraphPad Prism (Horsham, PA, USA). Within the text, data are expressed as mean plus or minus standard deviation. The Kolmogorov-Smirnov test was used to determine if data sets were parametric or non-parametric. One-way ANOVA followed by post hoc tests with Bonferroni correction for multiple testing was used when comparing continuous variables across multiple groups, where assumptions of normality were met. Otherwise multiple comparisons were carried out using the Kruskal Wallis test followed by Dunn's multiple comparison test. For the comparison of a numeric variable across two groups, Student's *t*-test was used where the assumption of normality was satisfied; otherwise the Mann-Whitney U test was applied. Spearman's rank correlation coefficient was used to assess the correlation between numeric variables. Within figures, the median is used to demonstrate which data sets are non-parametric, while the mean plus or minus the 95% confidence interval is used to demonstrate data sets which are parametric. P-values <0.05 were considered statistically significant.

Results

Fibrin formation and fibrinolysis in early-onset preeclampsia

A turbidometric based fibrin formation assay and immunoassays of tPA, PAI-1, PAI-2, plasmin-antiplasmin, and D-Dimer were used to assess and compare fibrin formation and fibrinolysis in non-pregnant controls, pregnant controls, and patients with early-onset preeclampsia. There was no significant difference in the lagtime to fibrin formation between non-pregnant controls, pregnant controls, and patients with early onset preeclampsia (Table 1). However, the maximum rate of fibrin formation (Vmax) was significantly increased in pregnant controls and patients with early onset preeclampsia compared to non-pregnant controls (Table 1); there was no significant difference between patients with early onset preeclampsia and pregnant controls (Table 1). Plasmin-antiplasmin levels were significantly lower in early onset preeclampsia and pregnant controls compared to non-pregnant controls (Table 1). However, there was no significant difference between early onset preeclampsia and pregnant controls. Levels of tPA were significantly increased in early onset preeclampsia compared to both non-pregnant controls and pregnant controls (Table 1). There was no significant difference in tPA between non-pregnant and pregnant controls. PAI-1 levels were significantly increased in early onset preeclampsia and pregnant controls compared to non-pregnant controls, but no difference between early-onset preeclampsia and pregnant controls (Table 1). PAI-2 levels were also significantly increased in EOP and pregnant controls compared to non-pregnant controls (Table 1). Again, there was no significant difference between early onset preeclampsia and pregnant controls. As expected, the ratio of PAI-1:PAI-2 was significantly lower in early onset preeclampsia and pregnant controls, compared to non-pregnant controls (Table 1). The ratio of PAI-1:PAI-2 was significantly lower in pregnant controls compared to EOP (Table 1). D-Dimer levels were significantly increased in EOP and pregnant controls compared to non-pregnant controls but there was no significant difference between patients with early onset preeclampsia and pregnant controls (Table 1).

Relationship between disease severity and fibrin formation/ fibrinolysis in early-onset preeclampsia

The presence of multiorgan involvement, the rate of disease progression (assessed as the time from diagnosis to time for need for delivery (days)), and the extent of the anti-angiogenic state measured (sFLT-1 & endoglin levels) were used to assess the effect of disease severity on fibrin formation and fibrinolysis. Within our early-onset preeclampsia cohort (n = 20), 11 cases were characterised by non-severe disease, while 9 cases were characterised by severe disease (multiorgan involvement). When comparing non-severe cases to severe cases, severe EOP patients had significantly increased plasma levels of tPA (Table 2) and D-Dimer levels (Table 2). There was a trend towards prolonged lagtime to fibrin formation in severe EOP cases. There was no significant difference in PAI-1, PAI-2, ratio of PAI-1:PAI-2, plasmin-antiplasmin, and Vmax of fibrin formation between non-severe and severe EOP cases (Table 2).

Table 2
Parameters of fibrin formation and fibrinolysis in non-severe and severe EOP (mean ± standard deviation).

	Non-severe EOP	Severe EOP	p-value
n	11	9	–
Lagtime to fibrin formation (seconds)	282.9 ± 97.3	351.7 ± 60.5	0.08
Vmax of fibrin formation	59.7 ± 27.3	71.8 ± 23.1	0.31
tPA (pg/ml)	5490 ± 3040	8665 ± 2296	0.02
PAI-1 (ng/ml)	84.5 ± 29.4	75.8 ± 27.3	0.28
PAI-2 (ng/ml)	114.4 ± 72.3	132.7 ± 53.8	0.55
PAI-1:PAI-2	1.1 ± 0.8	0.7 ± 0.3	0.16
Plasmin-antiplasmin(ng/ml)	422 ± 97.7	425.4 ± 139.3	0.94
D-Dimer (μg/ml)	3.4 ± 1.9	5.4 ± 2.2	0.03
sFLT-1 (pg/ml)	11,587 ± 4072	16478 ± 2992	0.007
Endoglin (pg/ml)	29.5 ± 10.8	38.6 ± 8.5	0.057

In cases of early onset preeclampsia, indicators that could prolong gestation prior to delivery have the potential to significantly improve fetal morbidity and mortality related to prematurity. As such, the time from early onset preeclampsia diagnosis to delivery is another indication of disease severity in this patient cohort. Within our EOP cohort, the time of diagnosis to delivery ranged from 0 to 7 days (1.1 ± 1.8 days, n = 20). Time from diagnosis to delivery significantly negatively correlated with tPA levels ($r = -0.578$, $p < 0.02$), D-Dimer levels ($r = -0.577$, $p < 0.03$), and the lagtime to fibrin formation ($r = -0.539$, $p < 0.05$).

Previous studies have shown that early onset preeclampsia is characterised by an anti-angiogenic state. sFLT-1 and endoglin are potent anti-angiogenic factors. Consistent with previous studies, our early onset preeclampsia cohort was characterised by significantly increased levels of both sFLT-1 and endoglin compared to healthy pregnant controls (Table 1).

There was a highly significant correlation between sFLT-1 and endoglin levels across our study cohort ($r = 0.913$, $p < 0.0001$). Soluble fLT-1 levels significantly correlated with the rate of fibrin formation ($r = 0.41$, $p < 0.05$), tPA ($r = 0.76$, $p < 0.05$), PAI-1 ($r = 0.75$, $p < 0.05$), PAI-2 ($r = 0.47$, $p < 0.05$), plasmin-antiplasmin($r = -0.32$, $p < 0.05$), and D-Dimer levels ($r = 0.72$, $p < 0.05$). Endoglin levels significantly correlated with the rate of fibrin formation ($r = 0.347$, $p < 0.05$), tPA ($r = 0.77$, $p < 0.05$), PAI-1 ($r = 0.74$, $p < 0.05$), PAI-2 ($r = 0.46$, $p < 0.05$), plasmin-antiplasmin($r = -0.34$, $p < 0.05$), and D-Dimer levels ($r = 0.68$, $p < 0.05$).

Discussion

In this study, we have simultaneously characterised multiple parameters of fibrin formation and fibrinolysis in a large prospective cohort of patients with early onset preeclampsia. This sample represents almost the entire EOP cohort seen over a two period in this centre, a large urban maternity hospital with almost 9000 live births per annum. The cohort is therefore highly representative of real-world clinical care.

EOP is associated with significant maternal and fetal morbidity and mortality. Delivery is the definitive treatment for preeclampsia and the safest option for the mother. However, premature delivery is associated with an increased risk of perinatal mortality and lifelong neurodevelopment complications for the fetus. Current guidelines recommend expectant management for EOP if both mother and fetus remain stable until 34 weeks. However, the commonly used criteria for severity (including hypertension and proteinuria) correlate poorly with maternal and fetal outcome [13]. Moreover, particularly in non-tertiary units, it is crucial that women with EOP are reliably and quickly identified to facilitate timely administration of steroids for fetal lung maturity, magnesium sulphate to prevent seizures and referral to specialized tertiary centres, as these measures can greatly reduce both maternal and fetal morbidity by facilitation of early intervention before severe complications have occurred [12,14–16]. As such, characterization of laboratory markers with differential expression in EOP and normal pregnancy is clinically relevant as this may uncover future biomarkers with diagnostic potential in this dangerous condition.

We report that there are major alterations in fibrin formation and fibrinolysis in early onset preeclampsia, most significantly in cases of severe disease.

During normal pregnancy, overall fibrinolytic activity decreases. Fibrinolysis activators t-PA and u-PA gradually increase, balanced by increased levels of PAI-1 (a key regulator of fibrinolysis *in vivo* [10,11]) and in late pregnancy, PAI-2 [10,17,18]. Certain markers of fibrinolytic turnover, including D-dimer, are however elevated, as is seen in our study [19]. Overall fibrinolytic activity is further decreased in preeclampsia relative to normal pregnancy [19]. Several studies have reported increased PAI-1 and t-PA [10,11,20,21] and decreased PAI-2 levels in preeclampsia compared with normal pregnancy [11,22]. Certain fibrinolytic parameters, namely an elevated PAI-1:PAI-2 ratio have shown promise in the prediction of both the clinical course of preeclampsia and degree of intrauterine growth retardation [23–26]. Our findings of altered fibrinolytic markers specifically in EOP with significantly decreased PAI-2 levels with an increased PAI-1:PAI-2 ratio are similar to those of Wikstrom *et al;* they found such alterations were unique to early onset preeclampsia when compared to a cohort of patients with late onset preeclampsia [18].

Our cohort contained 9 cases of severe early onset preeclampsia, that is disease associated with multiorgan involvement. tPA and d-dimer levels were significantly elevated in severe cases. This gives further weight to previous studies that have investigated tPA and d-dimer levels as predictors of disease severity in preeclampsia [22,23,25]. These markers, in addition to lagtime to fibrin formation, also correlated with disease progression. Their role as early predictors of disease progression and potential use for early identification of same has yet to be fully explored.

In this study, parameters of disease severity included the presence of multiorgan involvement, the rate of disease progression (assessed as the time from diagnosis to time for need for delivery (days)), and the extent of the anti-angiogenic state in this condition (a well-recognized marker of disease severity) [27]. Our findings of altered angiogenic markers in preeclampsia [28–31] and more specifically in EOP are in keeping with previous studies [32–35]. Verlohren et al reported that the sFLT-1:PLGF ratio permits identification of women with EOP at risk of imminent delivery [32]. Similarly, Goméz-Arriaga et al evaluated the ratio of sFLT-1:PLGF (in combination with gestational age at onset and uterine artery pulsatility-index) and demonstrated a significant association with perinatal complications and value in predicting timing of delivery in EOP [34]. More recently, the landmark PROGNOSIS study was a prospective, multicentre, observational study, in which serum sFLT-1:PLGF ratios were measured in 1050 women with suspected preeclampsia between 24 + 0 and 36 + 6 weeks' gestation. The authors derived and validated a serum sFLT-1:PLGF ratio that predicts lack of progression to preeclampsia in the subsequent week [35]. Our study confirmed significantly elevated s-Flt1 and endoglin levels in patients with EOP. In addition, levels correlated significantly with further alterations of coagulation and fibrinolytic markers in this patient group.

Potential limitations of this study included the fact that it was a single-centre study and that these findings, albeit representative of almost the entire prospective cohort of early onset preeclampsia cases in this large tertiary referral institution over a two-year period, will require confirmation in future larger multicentre prospective studies.

In conclusion, alterations of the coagulation and fibrinolytic system seen in healthy pregnancy are further amplified in early onset preeclampsia. Indeed certain alterations in markers of fibrin formation and fibrinolysis correlate with disease severity in this patient cohort.

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Author contributions

BH and FNA conceived and designed the study. HOC recruited patients. KP performed the experiments. KE, CM, and MG analysed the data. GH, KE, KP, HOC, CM, BK, PM, PBS, MG, JD, SA, NM, BH, and FNA wrote and edited the manuscript. The final submission was approved by all authors.

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