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Placental morphology, apoptosis, angiogenesis and epithelial mechanisms in early-onset preeclampsia

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ABSTRACT

Objectives: Early-onset preeclampsia is a form of preeclampsia requiring delivery before 34 weeks of gestation. The etiology is unknown, but placental dysfunction appears crucial. We evaluated the immunohistochemical expression of the antiapoptotic protein Bcl-2, the angiogenetic factors VEGF and PlGF, and the epithelial factors HGF, c-Met and STAT3 in placental samples of pregnancies complicated by early-onset preeclampsia.

Materials and methods: Placental sections were obtained from 41 women with early-onset preeclampsia (cases) and from 31 uncomplicated pregnancies (controls). A standard haematoxylin and eosin stain was used to assess histological structure. Immunohistochemical expression of Bcl-2, VEGF, PlGF, HGF, c-Met and STAT3 was analyzed.

Results: Mean gestational age was 32 weeks in cases and 39 weeks in controls. Microscopically, sections from women with preeclampsia showed a disorder of villous development as a distal villous hypoplasia with placental undergrowth. The immunoistochemical expression of Bcl-2 ($p < 0.0001$), VEGF ($p = 0.0323$), PlGF ($p = 0.002$), HGF ($p < 0.0001$), c-Met ($p < 0.0001$) and STAT3 ($p = 0.0004$) were significantly lower in placentas of complicated pregnancies compared to uncomplicated ones.

Conclusions: Early-onset preeclampsia is associated with a disorder of villous development. Apoptotic, angiogenetic and epithelial mechanisms are simultaneously impaired and contribute to placental dysfunctions.

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Introduction

Preeclampsia (PE) complicates about 2–8% of pregnancies, and is a major cause of maternal and perinatal morbidity and mortality [1]. PE impairs functioning of the kidneys, liver, and central nervous system [1]. Early preeclampsia is defined by the presence of a severe form of PE requiring delivery before 34 weeks of gestation [2]

Although the etiology of PE remains poorly understood [1–30], it is hypothesized that placental dysfunction may have a crucial role [31,32]. A common phenomenon observed in PE is the abnormal development and function of the placenta associated with an abnormal invasion and remodeling of maternal uterine arteries by extravillous trophoblasts [4]. Apoptotic, angiogenetic and epithelial mechanisms seem to be involved in placental insufficiency.

Exaggerated placental apoptosis is a common feature in PE [5]. B-cell lymphoma/leukemia (Bcl-2) is specifically considered as an important antiapoptotic protein and has been shown to prevent apoptosis by blocking cytochrome C release from mitochondria [6]. Bcl-2 may be downregulated in placentas from patients with PE [5].

Vascular Endothelial Growth Factor (VEGF) and the Placental Growth Factor (PlGF) are major factors regulating the placental angiogenesis for an adequate placental development and function. Defective action of these angiogenetic factors appears to play a major role in PE [3].

Epithelial growth factors are also involved in proliferation of trophoblastic cells. Among these, Hepatocyte Growth Factor (HGF), its receptor c-Met and the transcriptional factor STAT3 play an

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important role in regulation of normal fetal and placental development [7,8]. HGF is a cytokine that is able to generate, in cMET expressing cells, the branching morphogenesis that involves cellular changes in cell shape and cell polarization, cellular migration and cellular proliferation, creating the tridimensional branching extension [9]. A defective expression of HGF/c-Met/STAT3 pathway appears characteristic of organs and placentas from malformed fetuses [7,8].

The placental expression of antiapoptotic, angionenetic and epithelial growth factors normally increases during pregnancy until about 30 weeks with placental development, and then gradually decreases with placental aging [33,34]. By definition, early-onset PE requires delivery before 34 weeks of gestation, when the activity of these factor should be near its peak. We hypothesized that such process might be impaired in early-onset PE, and that the expression of these factor might be lower than in uncomplicated pregnancies.

The aim of our study is to evaluate the immunohistochemical expression of Bcl-2, PlGF, VEGF HGF, c-Met and STAT3 in placental samples from women affected by early-onset PE compared with uncomplicated pregnancies.

Materials and methods

Study protocol

The study protocol including methods for collection, analysis and interpretation of data was defined *a priori*. The study was designed as a retrospective case-control study and was reported following the STROBE guidelines [10].

Clinical data of patients were obtained by reviewing medical records of the Department of Neuroscience, Reproductive Sciences and Dentistry of the University Federico II, Naples, Italy. Data about gross and histologic examinations of placental specimens were retrieved from electronic databases of the Anatomic Pathology Unit of the Department of Advanced Biomedical Sciences, University Federico II, Naples, Italy. Paraffin-embedded specimens were retrieved from the archives of the same unit to perform *ad hoc* histomorphologic reviews and immunohistochemical analyses.

Cases

Diagnosis and management of PE was based on ACOG guidelines [1]. PE was defined as a blood pressure elevation ($\geq$ 140/90 on two occasions four hours apart or $\geq$160/110 once), after 20 weeks of gestation, with proteinuria ($\geq$300 mg on 24 h protein or >0.3 protein/creatinine ratio) or any of the following if proteinuria not presents: platelets <100,000; creatinine >1.1 (or doubling of creatinine in absence of other renal disease); doubling of AST or ALT. PE with severe features was defined as preeclampsia with any of the following: blood pressure $\geq$160/110 four hours apart on bed rest (unless on antihypertensive); platelets <100,000; doubling of AST or ALT; creatinine >1.1 (or doubling of creatinine in absence of other renal disease); pulmonary edema; new cerebral or visual disturbances. Early-onset PE was defined as PE requiring delivery before 34 weeks of gestation.

For this study we included women with singleton gestations and with early-onset PE without fetal malformations. We included all consecutive cases from March 2009 to April 2011

We selected as cases only women affected by early-onset PE, since it is considered as a distinct disease from late-onset PE [1], and is characterized by higher risk of fetal and perinatal morbidity/mortality [2]. To understand whether HGF/c-Met/STAT3 pathway deficiency characterizes PE independently from fetal malformations, we included in our study only placentas from fetuses without major congenital malformations.

Table 1
Characteristics of study population.

	Cases n = 41	Controls n = 31
Gravidity		
Median (25-75%; range)	1 (1–2; 1–7)	2 (1–3; 1–4)
Parity		
Median (25-75%; range)	0 (0–0; 0–2)	1 (0–2; 0–3)
Gestational age (weeks)		
Average ± SD	32 ± 2,4	39 ± 1,2
Maternal age (years)		
Average ± SD (range)	316 ± 4,4 (23–41)	335 ± 1,2 (23–41)
Race%		
White n (%)	41 (100)	31 (100)
Ethnicity		
Caucasian n (%)	41 (100)	31 (100)
Prenatal medications		
Iron n (%)	20 (50)	15 (484)
Vitamine and/or folic acid n (%)	41 (100)	31 (100)
Antihypertensive drugs n (%)	41 (100)	0 (0)
Smoking (before pregnancy)		
Cigarettes n (%)	5 (122)	0 (0)
Previous prenatal admission(s)		
Yes n (%)	0 (0)	0 (0)
Antibiotics in labor		
None n (%)	41 (100)	31 (100)
Beta strep status		
Positive n (%)	12 (293)	11 (355)
Antenatal steroids:		
Yes n (%)	41 (100)	0 (0)
GA mean ± SD	31 ± 2	0 (0)
Magnesium sulfate		
Yes n (%)	0 (0)	0 (0)
Anesthesia		
Epidural n (%)	41 (100)	31 (100)
Cervical ripening agent		
None n (%)	41 (100)	31 (100)
Labor		
Yes n (%)	0 (0)	0 (0)
Delivery mode		
C-section n (%)	41 (100)	31 (100)
Maternal Oxygen given at delivery?		
Yes n (%)	0 (0)	0 (0)
Birth weight (grams)		
Average ± SD	1541 ± 800	2900 ± 300
Placental weight (grams)		
Average ± SD	322 ± 103	556 ± 27
Baby's sex		
Female n (%)	25 (61)	18 (581)
Delivery to processing (mins)		
Average ± SD	30 ± 10,8	30 ± 9

Controls

Controls were low-risk pregnant women with uncomplicated singleton gestations. Intrauterine growth restriction (IUGR), chronic hypertension, diabetes, renal diseases were excluded. Controls were randomly selected in the same period as the cases.

For both cases and controls, gestational age had been assessed by the last menstrual period and confirmed by ultrasound.

Placental specimens

Placentas had been collected after a caesarean section and fixed for 24 h in 4% phosphate-buffered formaldehyde (Ph 7.2). Weight and diameter had been measured for each specimen; umbilical cord and membranes were removed before weight measurement. Sampling protocol included seven full-thickness section of the chorionic plate (four central, approximately equally spaced, and three peripheral), two sections of the membranes and two sections of the cord. After that, the tissues had been embedded in paraffin (melting point 52 °C); 4 μm paraffin sections had been mounted on glass slides covered with 3-amino-propyl–ethoxy3 silane (Sigma,

Deisenhofen, Germany) and a standard 52 haematoxylin and eosin stain had been used to assess histological structure.

Immunohistochemistry

Immunohistochemistry was carried with polyclonal rabbit antibodies against: HGFα (H-145, sc-7949, 200 μg/ml, 1:50, Santa Cruz Biotechnology, Santa Cruz CA), c-Met (B-2, sc-8057, 200 μg/ml, anti h-met, 1:50, Santa Cruz Biotechnology), STAT3 (h-190, sc-7179, 200 μg/ml, 1:50, Santa Cruz Biotechnology), VEGF (A-20, sc-152, 100 μg/ml, 1:200, Santa Cruz Biotechnology, recommended for 189, 165 and 121 amino acid splice variants of VEGF-A), PlGF (C-20, sc-1880, 200 μg/ml, 1:50, Santa Cruz Biotechnology), and mouse monoclonal antibody against Bcl-2 (124, M0887, 200 μg/ml, 1:100, DAKO).

Briefly, sections were deparaffinised with xylene, rehydrated through a graded series of ethanol solutions to distilled water, PBS (*phosphate buffered saline*) was used for all subsequent washes and for antiserum dilution. Tissue sections were treated sequentially in 0.3% hydrogen peroxide absolute methanol for 30 min to quench the endogenous peroxidase activity. Slides were then incubated at 4 °C overnight. After three washes in PBS to remove the excess of antiserum, the slides were incubated with goat anti-rabbit biotinylated antibodies (Vector laboratories, Burlingame CA, U.S. A.) at 1:200 dilution in PBS 3% for 1 h, all slides were then processed by the ABC method (Vector laboratories) for 30 min at room temperature. Diaminobenzidine (Vector Laboratories) activated with 0.05% hydrogen peroxide was used as final chromogen and Mayer's haematoxylin was used as the nuclear counterstaining. Negative controls for each tissue sections were prepared by leaving out the primary antiserum and replacing it with normal rabbit serum. Positive controls were represented by vascular tumor (angioma), breast carcinoma, colon carcinoma. All samples were processed under the same conditions. Immunocytochemical evaluation was performed by localization (epithelial, endothelial mesenchymal) and by quantification of reactivity.

Analysis and interpretation of data

The main outcome considered was the immunohistochemical expression of Bcl-2, PlGF, VEGF HGF, c-Met and STAT3 in the placental specimens.

The markers expression was dichotomized into negative or positive, based on the number of reactive cells < or >10% respectively, from observation of 10 fields using 40x magnification. Intensity of immunohistochemical staining was scored as follows: 0 = complete absence of staining; 1 = weak intensity of staining; 2 = moderate intensity of staining; 3 = strong intensity of staining.

Histological and immunocytochemical evaluation were performed independently by an expert perinatal pathologist (MDA) and a second pathologist (AT), who were blinded to clinical data. Disagreements were resolved by discussion at a two-headed microscope.

Univariate comparisons between cases and controls were performed by using Fisher's exact test for two-tailed P value with $\alpha = 0.05$ significance level.

Statistic analysis were performed using SPSS 17.0 package (*SPSS Inc., Chicago, IL, USA*).

Results

Characteristics of women

Forty-one women were included in the cases group and 31 in the control group.

In cases and controls, the mean maternal age was 31.6 ± 4.4 years (range: 23–41) and 33.5 ± 1.2 years (range: 23–41), the gestational age was 32 ± 2.4 weeks and 39 ± 1.2 weeks, the median gravidity was 1 (range: 1–7) and 2 (range: 1–4), the median parity was 0 (range: 0–2) and 1 (range: 0–3), respectively. All women were white and Caucasian. Among cases and controls, only 5 cases (12.2%) were smokers prior to pregnancy. All women delivered by cesarean section and no newborns presented malformations.

Characteristics are reported in detail in Table 1, according to Nelson et al. [12].

Characteristics of placentas

In cases and controls, placental weight was 322 ± 103 and 556 ± 27 g and placental diameter was 12 ± 2 and 18 ± 2 cm, respectively.

Microscopically, all cases showed a disorder of villous development as a distal villous hypoplasia with placental undergrowth (Fig. 1). The distal villi were decreased especially in the center of the placental lobule. Residual distal villi were sparse, thin, non-branching, poorly vascularized, with focal increase in the number of syncytial knots (Tenney-Parker change). The tertiary stem villi were prominent at the periphery of the lobule, they showed increased stromal collagen, muscular hypertrophy of arterioles, intervillous fibrin and villous agglutination. There was focal hypertrophic decidual vasculopathy.

On the other hand, no morphologic abnormalities were observed in controls' placentas.

Immunohistochemical findings

Where positive, the expression of VEGF, PlGF and Bcl-2 was localized in cytotrophoblastic cells. Negative expression of VEGF, PlGF and Bcl-2 was observed in 14 (34.1%), 19 (46.3%) and 33 (80.5%) cases respectively, and in 3 (9.7%), 3 (9.7%) and 0 (0%) controls respectively. Negative expression was significantly more

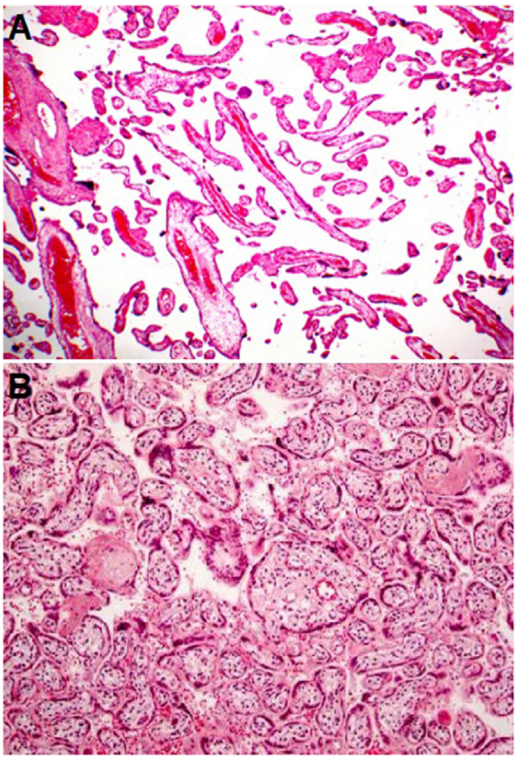

Fig. 1. Alteration of morphology of chorionic villi in women with early preeclampsia: accelerated maturation (A) and immaturity (B).

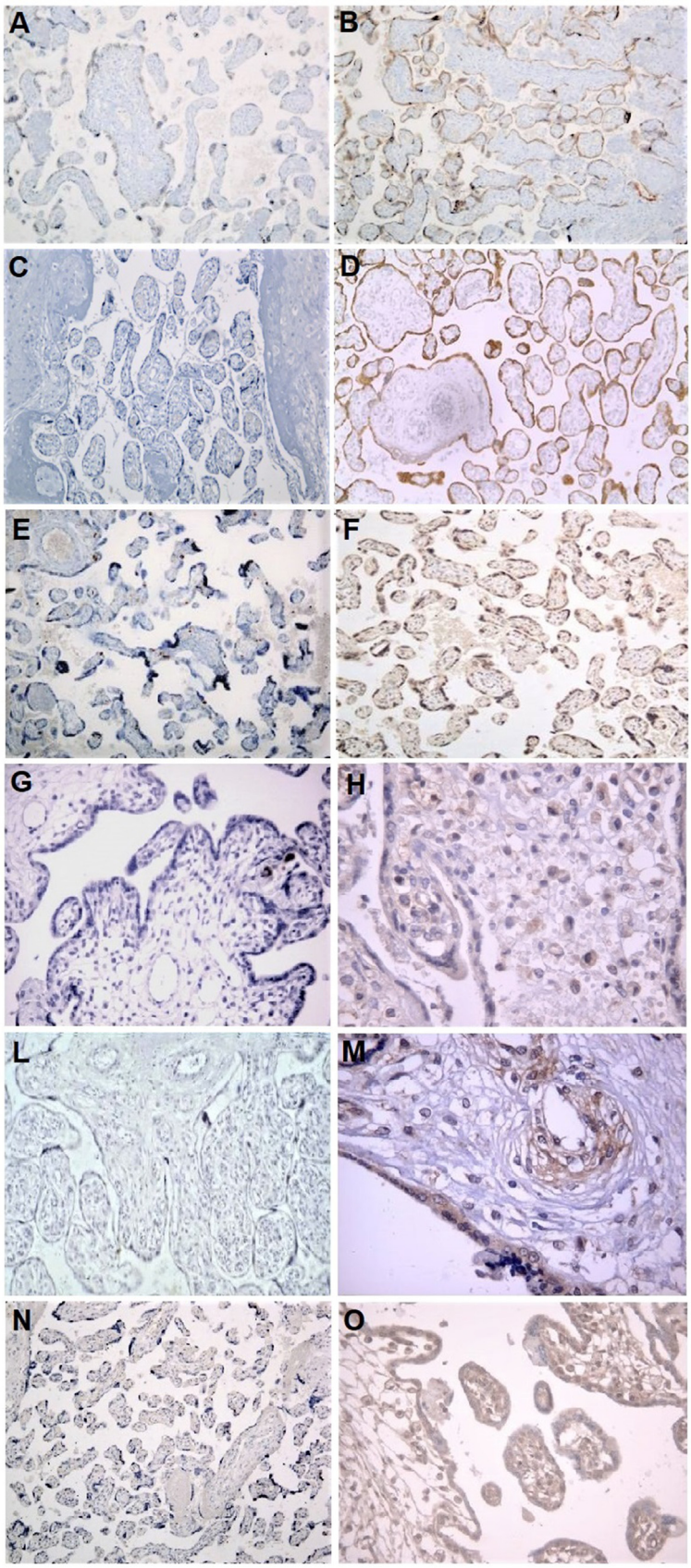

Fig. 2. Immunohistochemical expression of: Bcl-2 in cases (A, 100X) and controls (B, 40X); VEGF in cases (C, 40X) and controls (D, 40X); PlGF in cases (E, 40X) and controls (F, 40X); HGF in cases (G, 100X) and controls (H, 200X); c-Met in cases (I, 200X) and controls (L, 200X); STAT3 in cases (M, 40X) and controls (O,100X).

Table 2
Immunohistochemical expression of HGF, c-Met, Stat3, Bcl-2, VEGF, PlGF in cases and controls.

Marker	Negative expression		p-value
	Cases n (%)	Controls n (%)	
Bcl-2	33 (805)	0 (0)	<0.0001
VEGF	14 (341)	3 (9.7)	0.0323
PlGF	19 (463)	3 (9.7)	0.002
HGF	27 (65.8)	1 (3.2)	<0.0001
c-Met	26 (63.4)	1 (3.2)	<0.0001
Stat3	29 (707)	8 (25.8)	0.0004

common in cases than in controls for all markers (VEGF $p = 0.0323$; PlGF $p = 0.002$; Bcl-2 $p < 0.0001$)

Where positive, the expression of HGF, c-Met and STAT3 was localized in the cytoplasm of epithelial cells, fibroblast and myofibroblast cells. Negative expression of HGF, c-Met and STAT3 was observed in 27 (65.8%), 26 (63.4%) and 29 (70.7%) cases respectively, and in 1 (3.2%), 1 (3.2%) and 8 (25.8%) controls respectively. Negative expression was significantly more common in cases than in controls for all epithelial markers (HGF $p < 0.0001$; c-Met $p < 0.0001$; STAT3 $p = 0.0004$) (Fig. 2).

Immunohistochemical findings are summarized in Tables 2 and 3.

As expected, significant direct correlation was observed amongst epithelial factors (HGF, c-Met and STAT3); Bcl-2 and PlGF directly correlated with epithelial factors as well. Significant inverse correlation was found between the two angiogenic factors VEGF and PlGF (Table 4).

Discussion

Our study showed that a concomitant alteration in the expression of apoptotic, angiogenetic and epithelial factors is involved in placental dysfunctions underlying PE, independently from fetal malformations and with specific regard to early-onset PE.

Table 3
Intensity of immunohistochemical expression of HGF, c-Met, Stat3, Bcl-2, VEGF, PlGF in each case and control.

CONTROLS							CASES						
N	Bcl-2	VEGF	PlGF	HGF	c-Met	STAT3	N	Bcl-2	VEGF	PlGF	HGF	c-Met	STAT3
1	2	3	2	3	2	3	1	2	3	1	0	1	1
2	3	2	3	3	2	2	2	2	2	3	2	2	2
3	3	2	3	2	3	1	3	2	2	2	3	2	3
4	3	3	3	3	2	1	4	0	2	2	3	2	2
5	3	3	3	3	2	1	5	0	2	1	0	1	0
6	2	2	2	3	2	2	6	1	1	3	3	2	2
7	2	2	3	2	3	2	7	2	1	2	3	2	3
8	3	2	3	3	3	3	8	0	1	1	0	0	0
9	3	3	3	3	3	2	9	1	2	0	0	1	1
10	2	2	2	3	2	2	10	0	1	2	1	1	1
11	2	2	3	3	2	3	11	1	2	1	0	1	1
12	2	2	3	3	2	2	12	0	3	1	0	1	1
13	3	2	3	3	2	2	13	1	1	3	3	2	3
14	3	3	2	3	3	2	14	2	1	2	1	2	2
15	2	2	2	2	2	1	15	0	3	1	0	1	1
16	2	3	2	2	2	2	16	1	1	2	2	1	1
17	3	3	2	3	2	3	17	1	2	1	2	1	1
18	3	1	3	3	2	1	18	1	1	2	1	1	1
19	2	1	3	2	2	2	19	1	1	2	1	1	1
20	2	2	3	2	2	2	20	1	3	1	0	1	1
21	2	1	3	2	2	1	21	2	2	3	2	2	2
22	2	3	0	3	3	3	22	2	2	2	2	2	1
23	3	3	0	2	2	2	23	0	1	2	2	2	2
24	3	3	1	3	2	2	24	0	2	1	1	1	1
25	2	1	3	3	2	3	25	0	1	2	2	1	1
26	3	2	2	2	3	2	26	0	2	2	1	1	1
27	3	2	2	2	2	2	27	1	2	2	1	1	1
28	2	1	3	3	3	2	28	1	2	2	2	2	2
29	3	2	3	2	3	1	29	0	2	2	1	1	2
30	2	2	3	3	3	2	30	1	3	1	1	2	1
31	2	3	3	1	1	1	31	0	3	0	0	0	0
							32	1	2	2	1	2	1
							33	0	2	0	0	0	0
							34	1	1	2	2	2	1
							35	1	3	1	1	1	1
							36	0	3	1	0	1	1
							37	2	2	1	1	2	2
							38	0	3	0	0	1	1
							39	1	1	2	1	1	1
							40	0	1	1	1	1	1
							41	1	2	1	1	1	1

Table 4
P-values of the statistical association amongst the markers assessed.

	Bcl-2	VEGF	PIGF	HGF	c-Met	STAT3
Bcl-2	-	0.6925	0.2488	0.0966	0.0018*	0.0042*
VEGF	0.6925	-	0.0038**	0.1703	0.4816	0.7186
PIGF	0.2488	0.0038**	-	0.0003*	0.0028*	0.002*
HGF	0.0966	0.1703	0.0003*	-	0.0001*	0.0008*
c-Met	0.0018*	0.4816	0.0028*	0.0001*	-	<0.00001*
STAT3	0.0042*	0.7186	0.002*	0.0008*	<0.00001*	-

*: significant direct correlation; **: significant inverse correlation.

PE is often accompanied by hypoxia and reperfusion of the placenta, and this condition may induce apoptosis in trophoblastic cells. This hypoxia-induced apoptosis is accompanied by decreased expression of Bcl-2. Increased apoptosis together with decreased invasion and migration ability of trophoblast cells in the placenta have been proposed as a major causes of PE [5].

Endothelial dysfunction, caused by suppression of VEGF signaling, also seems to be a major factor involved in PE. VEGF and PlGF are angiogenetic factor which promote pseudovasculogenesis, *i.e.* the transformation on cytotrophoblast from an epithelia into an endothelial phenotype. Secretion of VEGF and PlGF is normally induced in trophoblast cells. In PE, increased level of the soluble fms-like tyrosine kinase 1 (sFlt-1) led to a suppression of VEGF and PlGF signalling, which may partially explain the vascular remodeling disorder described in patients with preeclampsia. Circulating levels of PlGF have been found decreased in women with severe preeclampsia comparing with normotensive pregnant [3].

Regarding epithelial factors, the cascade of HGF/c-Met/STAT3 plays a key role in proliferation and morphogenesis of many cells and tissues, including trophoblast. HGF is a pleiotropic mesenchyme-derived cytokine produced by fibroblasts and in general by mesenchymal cells, promoting hepatocyte proliferation *in vitro* [13]. HGF, binding c-Met, leads to dimerization, autophosphorylation and transient association of the receptor with cytoplasmatic STAT3 protein. This association induces STAT3 phosphorylation followed by translation to the nucleus [14]. STAT3 induces branching formation, cell migration, cell proliferation and morphogenesis of the trophoblast [15,16]. Also, it has been demonstrated that insufficient HGF production leads to kidney fibrosis [17]. Preeclampsia is associated with characteristic glomerular lesions, known as glomerular endotheliosis, and it appears that proteinuria in PE is the result of direct injury to renal podocytes [18]. Thus, it is presumable that HGF/cMet may play a role in the renal dysfunction of patients with PE.

In our series, all the studied factors showed a significantly lower expression in complicated pregnancies than uncomplicated ones. This result appears even more interesting if we consider that the expression of these molecules gradually decreases after about 30 weeks with placental aging, and thus it should have been higher in cases (mean gestational age = 32 weeks) than in controls (mean gestational age = 39 weeks). Also the placental morphology showed important differences between the two groups: cases showed a disorder of villous development as a distal villous hypoplasia with placental undergrowth, while the controls showed no abnormalities. Placenta with distal villous hypoplasia is unable to absorb oxygen. This pathologic aspect could result in placental dysfunction and abnormal feto-maternal flows [19].

Our sample may be the largest in the literature assessing c-Met and STAT3, and the second largest assessing HGF [20]. Moreover, this is the largest study evaluating c-Met, STAT3 and HGF specifically in early-onset PE (<34 weeks), which is considered as a condition distinct from late-onset PE [2].

To our knowledge, our study is the first to assess all these apoptotic, vascular and epithelial factors together, showing that the three mechanisms are simultaneously involved in the placental dysfunction underlying PE. Such a finding is consistent with scientific evidence on the interactions between these factors. Indeed, VEGF can inhibit apoptosis by inducing expression of Bcl-2 [21], and STAT3 protein is also implicated in the regulation of VGFR expression [22].

Another strength of our study was the collection of the analyzed tissues. In fact, the investigated cases included no malformed fetuses' placentas in pregnancies complicated by early-onset PE. This allowed to eliminate a possible confounding factor, as deficiency of HGF/c-Met/STAT3 pathway has been shown in organs and placentas from malformed fetuses [7,8].

On the other hand, the major limitation of our study may be the lack of matching between cases and controls with regard to some characteristics, *e.g.* gestational age. However, as discussed above, the difference in the gestational age should not affect our results, but rather it might even strengthen them.

Further studies are necessary to clarify etiopathogenetic mechanisms underlying early-onset PE, and to eventually identify novel diagnostic and therapeutic targets.

Conclusion

Early-onset PE is associated with a disorder of villous development, as a distal villous hypoplasia with placental undergrowth. Apoptotic, angiogenetic and epithelial mechanisms are simultaneously involved in placental dysfunctions underlying early-onset PE, as the expression of Bcl-2, PlGF, VEGF HGF, c-Met and STAT3 appeared significantly decreased in placentas from women with preeclampsia. Such association is independent from fetuses' malformations.

Disclosure of interest

The authors report no conflict of interest.

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