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Highlighting the R1 and R2 VEGF receptors in placentas resulting from normal development pregnancies and from pregnancies complicated by preeclampsia

MIHNEA ISTRATE[1)], CARINA MIHU[1)], SERGIU ŞUŞMAN[2)], CARMEN STANCA MELINCOVICI[2)], ANDREI MIHAI MĂLUŢAN[3)], RAREŞ BUIGA[4)], SORANA DANIELA BOLBOACĂ[5)], CARMEN MIHAELA MIHU[2)]

[1)]*Medical Student, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*

[2)]*Discipline of Histology, Department of Morphological Sciences, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*

[3)]*Discipline of Obstetrics and Gynecology II, Department of Mother and Child, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*

[4)]*Discipline of Anatomic Pathology, Department of Morphological Sciences, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*

[5)]*Discipline of Medical Informatics and Biostatistics, Department of Medical Education, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*

Abstract

Preeclampsia (PE), a pathological entity characterized by hypertension and pregnancy-related proteinuria, is a medical condition of incompletely known etiopathogenesis. Placental defects and placental angiogenesis may be a cause of this condition. The main factor that controls angiogenesis in the early stages of placental development is vascular endothelial growth factor A (VEGF-A) and its two receptors, namely VEGFR-1 and VEGFR-2. This study analyzed the immunohistochemical (IHC) expression of the two VEGF receptors, R1 and R2, in pregnancies complicated by PE compared to pregnancies with a normal evolution. The pregnancies included into the study for the harvesting of placental tissue to be microscopically analyzed were divided into two groups: the group of physiological pregnancies (22 pregnancies) and the group of pregnancies complicated by preeclampsia (13 pregnancies). For the microscopic analysis, we used the Hematoxylin–Eosin (HE), Masson's trichrome and IHC stainings. The microscopic aspects of HE and Masson's trichrome stainings most commonly found in normal development pregnancies underlie the normal process of placental senescence. In the case of pregnancies complicated by PE, the microscopic analysis of the placentas revealed fibrinoid necrosis of the vascular wall, lipid-loaded endothelial cells, diffuse trophoblastic hypertrophy, microinfarctions, calcification areas, fibrin deposits, vascular-syncytial membrane surface reduction, basement membrane thickening. According to the established marker intensity score, the VEGFR-1 and VEGFR-2 receptors were more pronounced in the placentas resulting from pregnancies complicated by preeclampsia. The present study brings arguments that support the major regulatory role of VEGF-A and of the two receptors in the normal or pathological angiogenesis in the placenta, and implicitly in the pathogenesis of preeclampsia. Further studies are needed for a more comprehensive analysis of the stages in which these factors cause alteration of the placental angiogenesis and vasculogenesis processes, so that they can intervene effectively in the treatment or prevention of this disease.

Keywords: VEGFR-1, VEGFR-2, immunohistochemistry, preeclampsia.

Introduction

Preeclampsia (PE) is a pregnancy-related disorder that triggers heterogeneous manifestations in both the mother and the fetus [1]. The etiopathogenesis of this condition is still incompletely known, with several hypotheses being issued, which is why this disease is also called the "disease of theories" [2–6].

The diagnosis of this condition is based on criteria set by the *International Society for the Study of Hypertension in Pregnancy* (ISSHP). In this context, preeclampsia is considered to be moderate if the blood pressure values range between 140/90 mmHg and 160/110 mmHg, and severe, if they exceed 160/110 mmHg and the proteinuria is over 5 g/24 h. In severe preeclampsia, there may occur manifestations, such as: pulmonary edema, oliguria, thrombocytopenia, abnormal liver enzymes, persistent headaches, visual disturbances, etc. Fetal impairment, *i.e.*, the occurrence of intrauterine growth retardation (IUGR), depends on the gestational age at which signs of preeclampsia occur. The clinical manifestations of PE become apparent in the second half of the pregnancy, but the pathophysiological processes are initiated in the first half of the pregnancy [7].

Many possible factors that may be involved in the occurrence of PE have been studied. It has been noted that the clinical manifestations of PE remit after the delivery of the placenta, and that pregnancy-induced hypertension also occurs in the case of hydatidiform mole, which is why the occurrence of this disease is considered to be directly related to the existence of placental tissue. Moreover, postpartum uterine curettage causes the blood pressure values to return to normal more quickly than in the absence of this procedure [8].

In this context, we believe that the placenta is a key element in the occurrence of PE, which is why placental and placental angiogenesis defects may be a cause of this disease. A primitive placental origin of arterial

hypertension in pregnancy is therefore possible, due to a placental angiogenesis defect, which will result in the failure of physiological vascular remodeling, with a reduction in the ratio between the vascular surface and the luminal diameter. The main factor that controls angiogenesis in the early stages of placental development is the vascular endothelial growth factor A (VEGF-A) and its two receptors: VEGFR-1 and VEGFR-2. Therefore, VEGF plays an important role in the formation of new blood vessels, in the proliferation, migration and metabolic activity of the trophoblast [9]. It is assumed that an imbalance between the angiogenic and antiangiogenic factors may lead to the occurrence of hypertension in pregnancy. In this study, we examined the relationship between the expression of the two R1 and R2 VEGF receptors and angiogenesis phenomena in normal pregnancies and in pregnancies complicated by PE.

Patients, Materials and Methods

Tissue collection

The study was conducted in the period between February 1 and December 1, 2016, at the "Dominic Stanca" Obstetrics and Gynecology Clinic, Cluj-Napoca, Romania.

The selection of pregnant women included in the study was carried out on a voluntary basis. The pregnant women enrolled in the study signed an informed consent. The study was endorsed by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca. The pregnancies included into the study for the harvesting of placental tissue to be microscopically analyzed, were divided into two groups: the group of physiological pregnancies (22 pregnancies) – the control group, and the group of pregnancies complicated by preeclampsia (13 pregnancies). Pregnancy evolution was monitored from 22 to 38 weeks of pregnancy by morphology ultrasound, Doppler ultrasound and three-dimensional (3D) power Doppler ultrasound, with the determination of placental vascular indexing. Two-dimensional (2D), 3D and Doppler ultrasound examinations were performed using an ACUVIX V10 ultrasound, in accordance with the established pregnancy monitoring protocol. The examinations were performed by an Obstetrics–Gynecology consultant.

Morphological study of placentas

The placentas were macroscopically and microscopically examined at the Department of Histology of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, as well as at the "Santomar" Medical Centre in Cluj-Napoca. The microscopic morphological examinations were performed using a DM 750 Leica microscope, equipped with an ICC50 HD Leica camcorder.

The placenta was harvested from natural or Caesarean section deliveries. Pregnancies complicated by PE have been defined in accordance with the standard *ISSHP* criteria. The macroscopic examination highlighted comparatively the weight, thickness and diameter of the placenta, as well as any possible anatomopathological changes in the surface or color of the placenta, in the two studied groups. The villous parenchyma has been inspected by sectioning the placenta at 1–2 cm intervals, looking for irregularities that could indicate infarction, thrombi, or other pathological entities.

Microscope slide preparation

Four pieces of villous parenchyma were harvested and placed in formalin fixative (10% neutral formalin). The volume of the fixative was 10 times the size of the fixed piece. The fixation time was 5–6 days. The inclusion was made in paraffin. The samples thus obtained were cut at 5–6 μm. For the microscopic study, we used the Hematoxylin–Eosin (HE) and Masson's trichrome stainings, in order to assess the normal or pathological aspect of the placenta. For immunohistochemical (IHC) staining, the sections on silane slides were incubated at 37°C (24 h). This was followed by dewaxing in xylene, rehydration in absolute ethyl alcohol, washing with distilled water, then Tris buffered saline (TBS). Antigen display was then achieved by boiling the sections in citric acid pH 6, followed by cooling at room temperature and washing in TBS. The following steps involved blocking peroxidases with hydrogen peroxide (Peroxidase Block 3–4%) and washing in TBS; blocking non-specific reactions with Protein Block and washing in TBS; incubating the sections with diluted primary antibody (VEGFR-1 and VEFR-2, respectively) in the wet chamber and washing in TBS (two consecutive baths). Post primary sensitization was performed to better penetrate the polymer, and the sections were incubated with NovoLink Polymer, a reagent containing the polymer-bound secondary antibody, and washing in TBS. The sections were incubated with 3,3'-Diaminobenzidine (DAB), washed with distilled water and counterstained with Hematoxylin. This was followed by washing under running water, with a saturated Lithium carbonate solution and rinsing with running water. The dehydration step in ethyl alcohol in increasing concentrations (75%, 85% absolute ethyl alcohol) was followed by xylene clarification (two consecutive baths) and mounting. Each case was assessed in terms of percentage and marker intensity. Before the assessment, the marker intensity was established on a scale from 1 to 3 (1 – weak, 2 – moderate, 3 – intense). The marker expression was assessed by two specialists.

Statistical analysis

The study is a descriptive one, so the quantitative data were summarized as ranges and mean values. The qualitative data were expressed as absolute frequency and as relative frequency accompanied by the 95% confidence interval (CI) computed using an exact method [10]. Differences between frequencies were tested using the *Z*-test for proportion. The univariate scatterplot was used to illustrate the distribution of %R1 and %R2 intensity by groups [11]. The Mann–Whitney test was applied to identify if statistically significant differences in the intensity expression of investigated receptors exists between groups. A *p*-value of 5% was considered as threshold for significance. Statistical analysis was conducted with Statistica program (StatSoft, *v.* 8).

Results

Thirty-five women aged between 17 and 41 years, with a median of age equal to 30 years (interquartile range from 25.5 to 34 years) were included in the study. No significant differences between groups were identified

in regards of age (Mann–Whitney test: *Z*-score 1.14, p=0.2528).

In the studied sample, from 35 pregnancies, 21 babies were born by Caesarean section, all in the group with preeclampsia (100%) and six (27.27%, 95% CI [9.3–49.79]) among the controls.

All babies were term delivered, most of them at 40 weeks of gestation in the preeclampsia group, and at 39 weeks of gestation in the controls, without any significant differences between groups (p>0.12).

The macroscopic examination evaluated the weight, thickness and diameter of the placenta, as well as any possible anatomopathological changes in the surface or color of the placenta, in the two studied groups (localized areas of pallor caused by fetal artery thrombosis, infarction, retroplacental hematomas). Placentas from the group with preeclampsia were found to be smaller than those from normal pregnancies. The infarcted area was larger in placentas from preeclampsia compared to what was found in the placentas from uncomplicated pregnancies. The incidence of placental infarction was higher in placentas from pregnancies with preeclampsia (Figure 1, Table 1).

The most frequently noted microscopic aspects in normal development pregnancies were: trophoblastic degeneration, villous fibrillation stroma (through the transformation of the mesenchymal tissue into fibrillated connective tissue, as a part of the normal process of placental senescence), calcium deposits, vascular obliteration and fibrin deposits. Perivillous and intravillous fibrin deposition are common and are generally considered normal within the placenta. Calcification is other common phenomenon in the mature placenta. Third trimester gestation have an increase for calcium. Calcification of the placenta is a normal physiological response to the development and aging. These microscopic changes underline the normal placental senescence process, which also influences placental function (Figures 2 and 3).

In the case of the pregnancies complicated by PE, the microscopic analysis of the placenta revealed fibrinoid necrosis of the vascular wall (as a result of excessive deposition of fibrinoid), lipid-loaded endothelial cells, diffuse trophoblastic hypertrophy, microinfarctions (placental infarcts are a common lesion in PE), calcification areas (Figure 4a), vascular-synovial membrane surface reduction (as a response to the decrease of the vascularization in the terminal villi) (Figure 4b); basement membrane thickening (usually, the basement membrane can only be seen in special stains; in placentas from preeclampsia, the marked thickening of the basement membrane is clearly visible in HE staining), numerous syncytial buds (they can usually be found under hipoxic conditions) (Figure 4c), fibrin deposits (fibrinoid is an acellular intensely staining eosinophilic material that is mostly related to the intervillous space) (Figure 4d). In placentas from preeclamptic gestations appears the Tenney–Parker change. This change is characterized by an increased syncytial knotting on villi, considered to be failed adaptative responses to low oxygen tension within the intervillous space. In preeclamptic gestations, decidual vasculopathy is encountered.

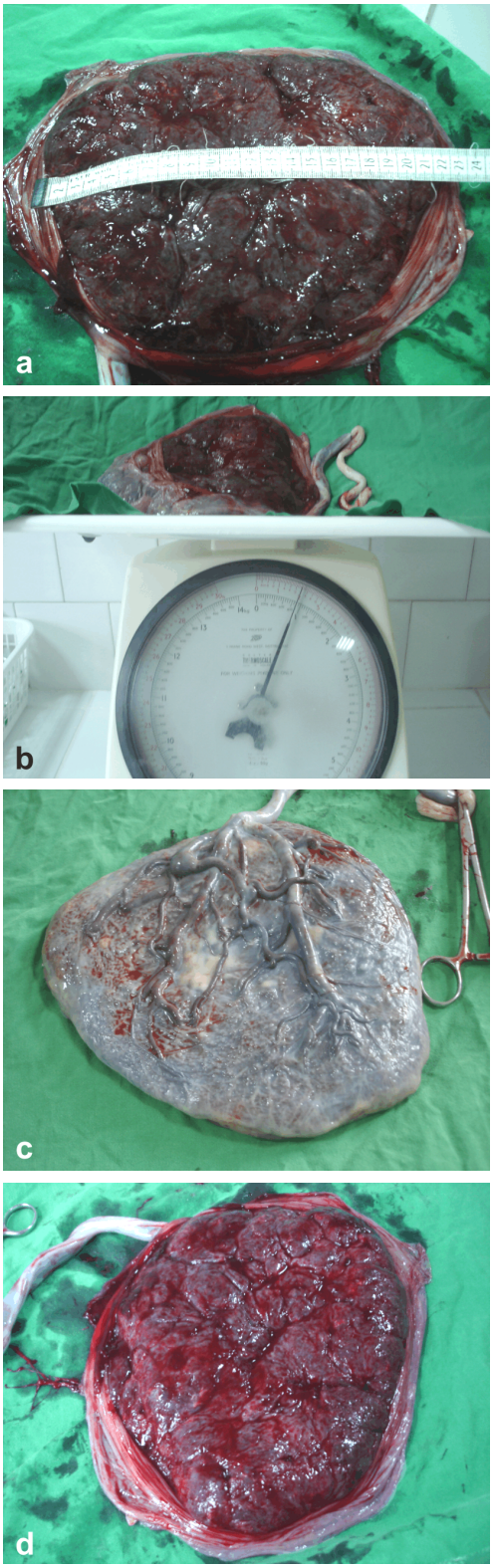

Figure 1 – *(a) Placental diameter, (b) weight, (c) fetal placental sides, (d) maternal placental sides.*

Table 1 – *Macroscopic examination of the placentas for the investigated groups*

Group	Interval of gestational age [weeks]	Average weight [g]	Average thickness [mm]	Average diameter [mm]
Control	38–42	500	35	190/180
PE	38–42	480	32	150/130

PE: Preeclampsia.

The assessment of the IHC expression of the two receptors, R1 and R2, was performed comparatively in the placentas resulting from normal pregnancies and from pregnancies complicated by PE. According to the established marker intensity score, the two receptors were more pronounced in the placentas resulting from pregnancies complicated by preeclampsia (Figure 5).

The % presentation of R1 and R2 at the level of T (trophoblast) and V (vascular endothelium) varied in the same ranges for both groups, without any significant differences between the groups (Figure 6).

Despite the fact that the intensity of the investigated receptors was visually different in the case of PE, as compared with the controls, with one exception represented by 1+ intensity of R1-V (less frequent in the PE group, see Table 2, $p<0.05$), no other statistically significant differences were identified (Table 2).

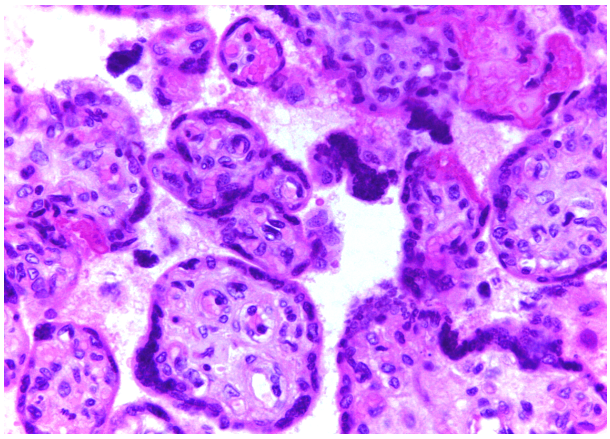

Figure 2 – ***Aspects of chorionic villi, with some fibrin deposits (HE staining, ×400).***

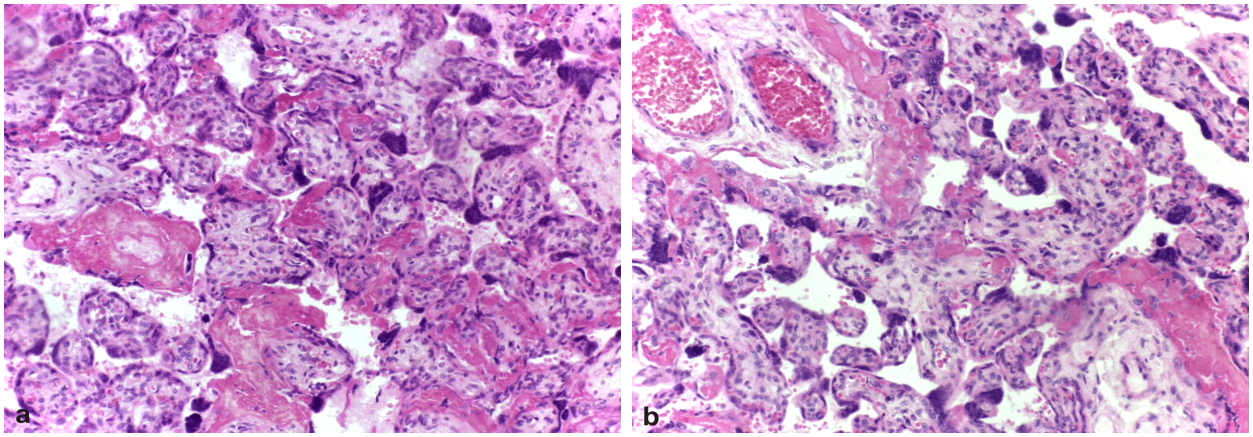

Figure 3 – ***Intravillositary (a) and perivillositary (b) deposits of fibrin (HE staining, ×200).***

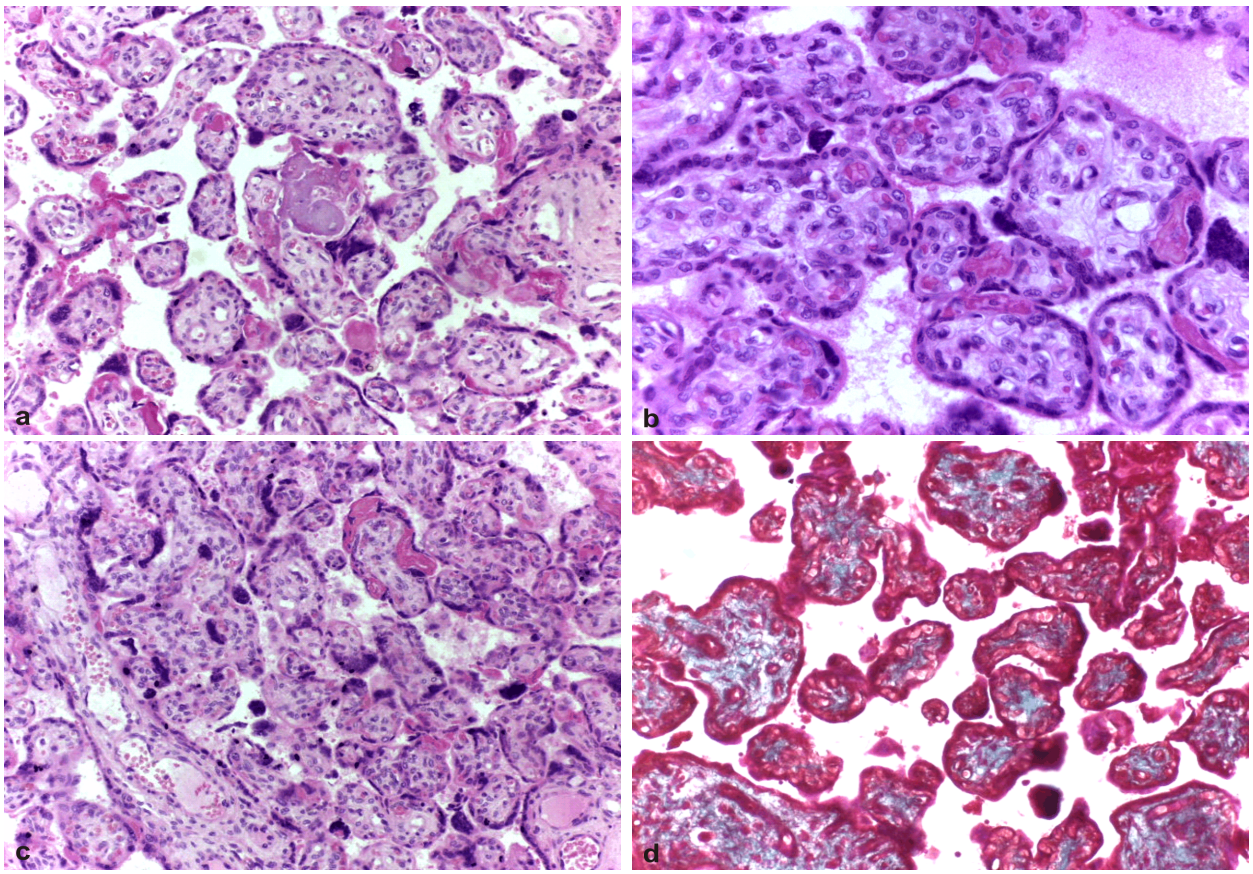

Figure 4 – ***(a) Calcifying areas; (b) Vasculo-syncytial membrane surface reduction; (c) Increased syncytial knotting on villi; (d) Fibrin deposits. HE staining: ×200 (a and c); ×400 (b). Masson's trichrome staining: ×200 (d).***

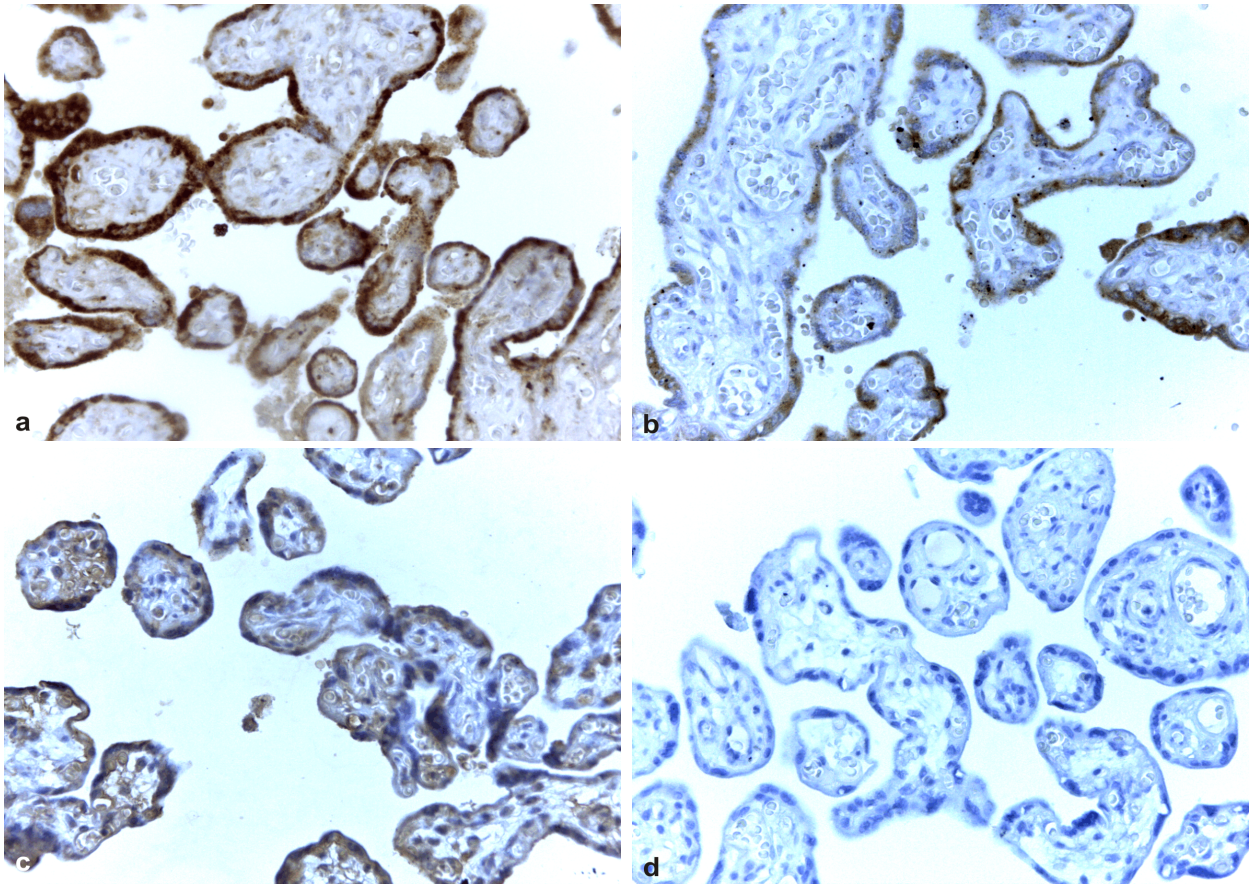

Figure 5 – *(a) VEGFR-1 strongly positive at the level of the trophoblast in pregnancies complicated by PE; (b) VEGFR-1 weakly positive at the level of the trophoblast in physiological pregnancies; (c) VEGFR-2 positive in the endothelium in pregnancies with PE; (d) VEGFR-2 very low positive in the endothelium in physiological pregnancies. IHC staining for VEGFR-1: (a and b) ×200. IHC staining for VEGFR-2: (c and d) ×200. VEGFR-1 and VEGFR-2: Vascular endothelial growth factor A (VEGF-A) receptors – R1 and R2, respectively; PE: Preeclampsia; IHC: Immunohistochemistry.*

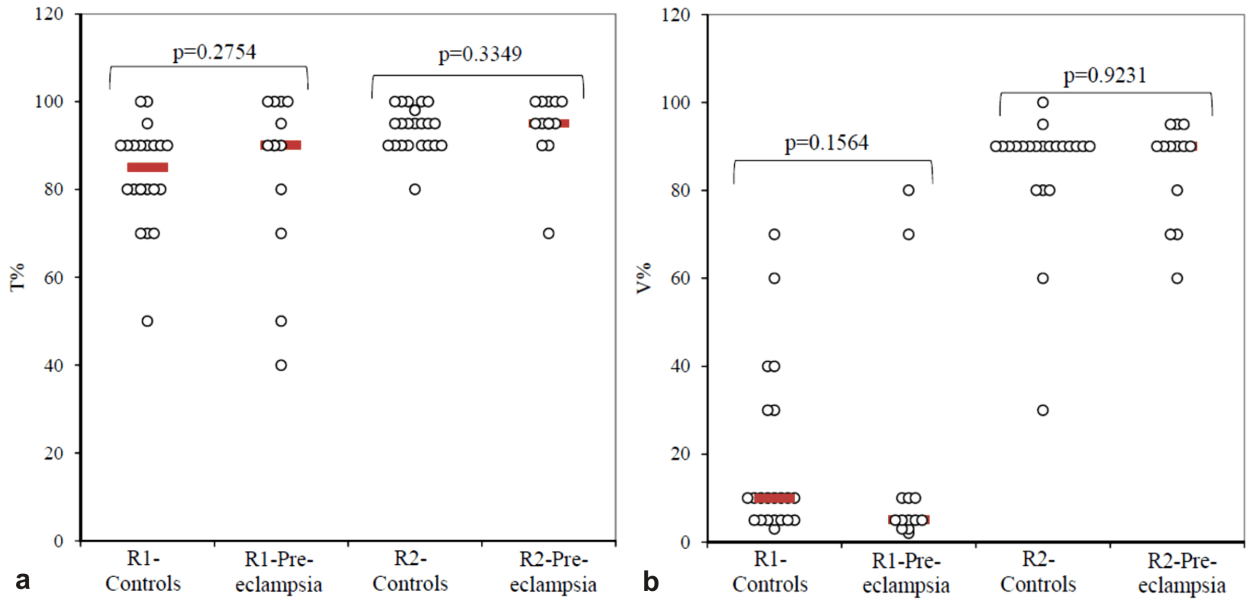

Figure 6 – *(a) %R1 T and %R2 T; (b) %R1 V and %R2 V. The dots are the individual values and the red line is the value of the median. T: Trophoblast; V: Vascular endothelium.*

Table 2 – ***Receptor expressions by group expressed as number and (%)***

Characteristic	Controls (*n*=22)	Preeclampsia (*n*=13)	*p*-value
R1-T			
1+	9 (40.91)	3 (23.08)	0.2907
2+	12 (54.55)	9 (69.23)	0.3978
3+	1 (4.55)	1 (7.69)	0.7015
R2-T			
1+	3 (13.64)	0 (0)	0.173
2+	10 (45.45)	6 (46.15)	0.9682
3+	9 (40.91)	6 (46.15)	0.764
R1-V			
1+	20 (90.91)	8 (61.54)	0.0436
2+	1 (4.55)	0 (0)	0.4408
Missing data	1 (4.55)	5 (38.46)	
R2-V			0.8872
1+	9 (40.91)	5 (38.46)	
2+	13 (59.09)	8 (61.54)	

T: Trophoblast; V: Vascular endothelium.

Discussions

In the past 10 years, there have been over 10 000 scientific articles on etiopathogenicity, as well as on the possibilities of diagnosis and treatment in preeclampsia. However, there are still many unlearned aspects of this condition. Only once clear etiology has been established for this pathology, can effective prophylaxis measures be taken. Three directions have been outlined in the pathogenesis of PE: placental defects, placental ischemia and endothelial dysfunction. Many of the studies are focused on the investigation of endothelial dysfunction [12–15].

The morphological development of the placenta is correlated with the development of vascular structures. In the initial stages of placental development vasculogenesis processes take place, *i.e.*, the formation of new blood vessels, starting from angioblasts. This is followed by the angiogenesis process, which will turn the placenta into an eminently vascular organ. Angiogenesis involves the branching of new microvessels from pre-existing larger blood vessels. VEGF-A and its receptors regulate placental vasculogenesis and angiogenesis throughout the embryogenesis. VEGF-A is the most important endothelial growth factor, stimulating angiogenesis and the proliferation of endothelial cells, and it is also an important factor in regulating vasculogenesis [16]. VEGF action is mediated by the binding to the two VEGF receptors, R1 and R2. VEGFR-1 inhibits the endothelial function, acting, most likely, as a barrier between the maternal and fetal circulation, suppressing angiogenesis and vascular permeability. VEGFR-2 stimulates the proliferation, migration and permeability processes of the endothelial cells. The two receptors thus interfere with the regulation of vasculogenesis and angiogenesis, and an alteration of the expression of these receptors causes the formation of aberrant blood vessels [17]. In PE and IUGR, studies report poor angiogenesis, which will cause placental ischemia and implicitly alteration of the fetal–maternal exchanges [18].

The literature data on the expression of VEGF in pregnancies with normal evolution compared to pregnancies complicated by PE are often contradictory. Many studies assess the serum level of VEGF in the maternal circulation, reporting increases or decreases in the serum level of VEGF in normal pregnancies *versus* pregnancies with PE [19–21]. Most studies correlate this increase in serum levels of VEGFR-1 with a risk of preeclampsia [22–24].

There are few studies in the literature that assess the IHC expression of VEGFR-1 and VEGFR-2 receptors in normal development pregnancies compared to pregnancies complicated by PE. In our study, VEGFR-1 expression was increased in the trophoblast and endothelial cells in placentas resulting from pregnancies complications by PE, which indicates abnormality in the VEGF-mediated function in placental cells, an aspect also supported by other studies [23, 25]. In this context, we can assume that VEGFR-1 is a key factor in the occurrence of PE, because an increase in expression of this receptor causes a change in the response of trophoblastic and endothelial cells, inhibiting endothelial proliferation and vasodilatation. Our study supports the claim that increased expression of VEGFR-1 is directly related to PE occurrence, which is consistent with other research conclusions [26, 27]. Many of the studies published in the literature report an increase of VEGFR-1 expression in preeclampsia, as highlighted by our study as well [28, 29]. There are also studies suggesting that VEGFR-1 expression in PE is low, and that it is not possible to determine whether this modification is the cause or consequence of the pathogenic status of pregnancy, low levels of VEGFR-1 in the placental bed may explain the defective uterine vascularization often associated with early-onset preeclampsia [30].

Given the current controversy over the role played by the two VEGF receptors in the occurrence of preeclampsia, we believe that the present study supports one of the most accepted theories on the pathogenesis of preeclampsia, that according to which there is an imbalance between the factors that mediate placental angiogenesis [31–33]. We can assert that a change in the expression of VEGF-A and of the two receptors determines the alteration of the angiogenesis process, an aspect also highlighted by the microscopic examination of the placentas, which showed dilated capillaries, capillary stasis and thrombosis in the terminal villi in the pregnancies that evolved with PE, which is consistent with the claims made by other studies in the literature [8, 34–36].

Conclusions

VEGFR-1 is essential for the normal development and function of the placenta. An imbalance between the angiogenic and antiangiogenic factors is determinant in

the occurrence of PE. The present study brings arguments that support the major regulatory role of VEGF-A and of the two receptors in the normal or pathological angiogenesis in the placenta, and implicitly in the pathogenesis of preeclampsia. Further studies are needed for a more comprehensive analysis of the stages in which these factors cause alteration of the placental angiogenesis and vasculogenesis processes so that they can intervene effectively in the treatment or prevention of this disease.

Conflict of interests

The authors declare that they have no conflict of interests.

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Corresponding author
Carina Mihu, Medical Student, Faculty of Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, 4–6 Louis Pasteur Street, 400349 Cluj-Napoca, Romania; Phone +40727–227 462, e-mail: carina.mihu@umfcluj.ro

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