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VEGF expression in the placenta from pregnancies complicated by hypertensive disorders

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Objective To determine the expression of VEGF in the placental tissue from pregnancies complicated by hypertension disorders of different clinical severity.

Design Prospective cohort study.

Setting Polyclinic of Careggi, University of Florence, Italy.

Sample Placentas from women with gestational hypertension ($n = 20$), pre-eclampsia ($n = 20$) and pre-eclampsia with HELLP syndrome ($n = 20$) and from normotensive women ($n = 20$), as control group (gestational age comprised between 35 and 38 weeks).

Methods An immunohistochemical technique and a quantitative analysis to measure mRNA levels (RT-PCR) were employed.

Main outcome measures Intensity of immunoreactivity and mRNA levels in the placental components. Differences between the data.

Results VEGF immunoreactivity was observable in all the placental components in the gestational hypertension cases as in the control ones. In the cases with pre-eclampsia and pre-eclampsia with HELLP syndrome, some placental components were not immunoreactive. However, the VEGF positive components of all the pathological groups showed a higher intensity of reactivity with respect to that of the control group. The levels of VEGF mRNA were higher in the gestational hypertension cases and lower in the cases of pre-eclampsia with HELLP syndrome with respect to the control ones; in the cases of pre-eclampsia, the levels were the same as the control ones.

Conclusion The different expression of VEGF in the placenta of the pathological cases is probably related to haemodynamic changes that take place in these disorders, in order to attempt restoration of a normal uteroplacental flow.

INTRODUCTION

In physiological pregnancies, successful placentation involves the development of a low-impedance uteroplacental circulation after trophoblast invasion and transformation of the maternal intramyometrial portion of the spiral arterioles.[1,2] In pregnancies complicated by pre-eclampsia, trophoblast invasion of the spiral arterioles is abnormal, resulting in impaired uteroplacental perfusion ('placental hypoxia' theory).[3–6] This in turn results in the release of factors into the maternal circulation that may be responsible in endothelial dysfunction, vasoconstriction and hypertension.[4,6,7]

Vascular endothelial growth factor (VEGF) is a disulphide-linked homodimeric glycoprotein that is selectively mitogenic for endothelial cells and appears to play a major role in the mediation of vasculogenesis, angiogenesis, in the control of the microvascular permeability and of the vasodilatation.[8–14] Hypoxia is a potent stimulus for induction of VEGF gene expression and has been shown to increase the stability of VEGF mRNA.[12,15,16] VEGF and other factors are likely to be involved in the uterine vessels remodelling and in the angiogenesis which occurs during the growth of the placenta throughout pregnancy.[10,17,18] Another important role has been postulated for VEGF in the regulation of the trophoblast invasion, proliferation and differentiation.[15,18–24] In the human placenta, this factor has chiefly been identified in the villous cytotrophoblast in the first trimester and in the syncytiotrophoblast and extravillous trophoblast in the term placenta.[8,10]

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Various studies have been carried out on the plasma or serum concentration of VEGF and its expression in the placental tissue of women with pregnancies complicated by hypertensive disorders. Several investigations have shown that VEGF concentrations are increased in the plasma or serum of women with pregnancies complicated by hypertensive disorders compared with normotensive women, although this is not confirmed in other studies.[25–34] Studies of the placental VEGF immunostaining in pregnancies complicated by more or less severe hypertensive disorders have reported a decrease of this factor, while in another study an increase has been observed.[26,31,35] Investigations on the mRNA of VEGF have shown that its levels were lower in pre-eclamptic placentas compared with control placentas[36]; other studies have demonstrated either an increase or no evidence of abnormal placental VEGF expression in pregnancies complicated by pre-eclampsia.[31,37]

The aim of this study was to evaluate the expression of the VEGF in placentas from pregnancies complicated by hypertensive disorders of different clinical severity, in an attempt to clarify these disagreements.

METHODS

Three study groups were considered: (1) a first study group of women with pregnancies complicated by gestational hypertension ($n = 20$); (2) a second study group of women with pregnancies complicated by pre-eclampsia ($n = 20$); (3) a third study group of women with pregnancies complicated by pre-eclampsia with HELLP syndrome ($n = 20$). Women with uncomplicated pregnancies ($n = 20$) were examined as control group. The gestational age was comprised between 35 and 38 weeks.

No women with chronic hypertension, renal disease or diabetes were included. Women in all the study groups were delivered by caesarean route. Controls were women undergoing cesarean section on one of the following indications: breech presentation, cephalopelvic disproportion or psychological reasons.

Gestational hypertension was defined as persistent diastolic blood pressure above 90 mmHg and systolic blood pressure above 140 mmHg in normotensive patients before 20 weeks of gestation. Criteria for diagnosis of pre-eclampsia were: diastolic blood pressure above 90 mmHg and systolic blood pressure above 140 mmHg, with oedema and proteinuria greater than 0.3 g/24 h in normotensive patients before 20 weeks of gestation. Criteria for diagnosis of the HELLP syndrome were: (1) haemolysis: abnormal peripheral blood smear, total bilirubin $\geq$1.2 mg/dL, lactic dehydrogenase (LDH) $\geq$600 U/L; (2) elevated liver enzymes: serum aspartate aminotransferase (AST) $\geq$70 U/L; (3) low platelets: platelets count $\leq$100,000/μL.

Informed written consent was obtained from each patient.

At delivery, the placentas were weighed and full thickness blocks were obtained. A stratified random sampling procedure was used to obtain 12 blocks of full thickness tissue per organ. Six specimens were immersed in neutral 4% buffered formalin solution for 12 hours and processed in a standard manner for the preparation of paraffin blocks; sections of 5 μm thickness were then obtained. The other specimens were snap frozen without delay in liquid nitrogen and stored in −80°C.

The sections were treated according to the standard immunohistochemical technique for detection of VEGF. Endogenous peroxidase was blocked by incubation of the sections in 0.3% hydrogen peroxide. The samples were incubated with blocking serum to reduce non-specific reactions and then incubated with the primary antibody. The VEGF antibody used was a monoclonal mouse anti-humane VEGF (Santa Cruz, California, USA) at a dilution of 1:80. The remaining procedure was performed using the mouse ABC kit Staining Systems (Santa Cruz, California, USA). Immunoreactive VEGF was localised by incubating sections in 0.7 mg/mL 3,3′-diaminobenzidine and 0.7 mg/mL hydrogen peroxide in 0.06 M TBS. Ten fields were examined for each section by an investigator blinded to the tissue identity and scored for location of staining in trophoblast, stromal cells, Hofbauer cells and vessels. All sections were stained in the same batch to eliminate inter-batch variations.

RNA extraction from the frozen specimens was performed by disruption in 600 μL of guanidine isothiocyanate-containing lysis Qiagen buffer (QIAGEN, Milan, Italy) added with β-merchaptoethanol. Total RNA was extracted with Rneasy MiniKit Qiagen columns and treated with Rnase free DNAse Set QIAGEN. RNA was then eluted from columns with 50 μL Rnase free water. Total RNA was determined with the Pharmacia GeneQuant spectrophotometer.

A standard RNA preparation for the proposed VEGF RT-PCR assay was obtained by a conventional phenol–chloroform extraction from V-12 cells, over-expressing VEGF. The amount of the extracted RNA was evaluated spectrophotometrically and then the concentration of the VEGF mRNA was accurately determined by a competitive RT-PCR assay. For this purpose, a synthetic internal standard was constructed by the oligonucleotide overlap extension technique. Details about this procedure have been previously described.[38,39] Aliquots of the VEGF mRNA for the standard curve were stored at −80°C. In each assay, a VEGF mRNA amount in the range 2.5×10^9–2.5×10^4 molecules was used for standard curve. Finally, results of the VEGF mRNA concentration were expressed in terms of VEGF mRNA molecules/μg total RNA.

All reagents both for reverse transcription and amplification were from Applied Biosystem (Foster City, California, USA). Fifty nanograms of extracted mRNA from the standard preparation and from human specimens were reverse-transcribed using the TaqMan Reverse Trascription

Table 1. Clinical details of the study groups. Values shown are mean (SEM), unless otherwise indicated.

	Control ($n = 20$)[a]	Hypertension ($n = 20$)[b]	Pre-eclampsia ($n = 20$)[c]	Pre-eclampsia/HELLP syndrome ($n = 20$)[d]
(1) Maternal age (years)	30.2 (7.4)	31.0 (8.3)	30.2 (5.8)	29.4 (6.1)
(2) Gestation at delivery (weeks)	38.0 (1.3)	37.8 (1.8)	35.1 (5.9)*	35.0 (4.0)*
(3) Birthweight (g)	3480.1 (610.1)	3310.4 (712.2)	2240.2 (318.1)*	2080.1 (428.2)*
(4) Placenta weight (g)	618.8 (80.1)	524.4 (59.1)	412.4 (64.3)**	385.2 (52.7)**
(5) Primigravidae (*n*)	12	10	10	12
(6) Smokers (*n*)	4	12	4	4
(7) Pregnancy BMI	22.4 (2.3)	24.8 (3.2)	23.5 (3.2)	24.1 (4.1)
Blood pressure at delivery (mmHg)				
(8) Systolic	111.2 (9.3)	148.8 (9.1)*	155.2 (10.3)*	147.2 (8.2)*
(9) Diastolic	65.7 (8.2)	97.3 (6.5)*	101.4 (10.1)*	97.4 (7.4)*

BMI = body mass index; (2) c, d *versus* a, b: *$P < 0.005$; (3) c, d *versus* a, b: *$P < 0.005$; (4) c, d *versus* a, b: **$P < 0.05$; (8) c *versus* b, d *versus* a: *$P < 0.005$; (9) c *versus* b, d *versus* a: *$P < 0.005$.

Reagents Kit. According to the kit instructions, reagents were prepared to give final concentration of 1× TaqMan RT-buffer, 2.5 mM random hexamers, 500 mM each dNTP, 5.5 mM $MgCl_2$ and 1.25 U/mL, Multiscribe TM reverse transcriptase in a final volume of 20 μL. Retrotranscription was performed using the following programme: 10 minutes 25°C, 30 minutes at 48°C, 5 minutes at 95°C. Subsequently, 5 μL of the RT sample was amplified. The PCR mix contained TaqMan Universal Master Mix, 300 nmol/L each primer and 200 nmol/L probe. VEGF mRNA was amplified by a first step of 2 minutes at 50°C, 10 minutes at 95°C followed from 40 cycles of 15 seconds at 95°C, 1 minute at 60°C. All the RT-PCR reactions were performed in the ABI PRISM 7700 Sequence Detector in a 25 μL final volume. Standard curve and samples were carried out in triplicates.

Primers for the real-time RT-PCR assay, selected using the proprietary software Primer Express (Applied Biosystem), were: 5′-TACCTCCACCATGCCAAGTG-3′ (forward primer), 5′-ATGATTCTGCCCTCCTCCTTC-3′ (reverse primer). The sequence of the fluorogenic probe (FAM) was: 5′-TCCCAGGCTGCACCCATGGC-3′.

To compare clinical data between the study groups, the one-way ANOVA and ANOVA for rank, when appropriate, were applied.

The *t* test for paired samples was used to test differences between the data on the VEGF mRNA concentrations of the control group and those of the other study groups. Probability of less than 0.05 ($P < 0.05$) was considered statistically significant.

For each section VEGF immunostained, a statistical analysis was also performed to evaluate the intensity of immunoreactivity in the trophoblast, in the wall vessels, in the stromal cells and in the Hofbauer cells. For this purpose, the optical density was measured on grey scale images using a computerised image analyser programme (Image-Pro Plus v. 4.5, Media Cybernetics, Silver Spring, Maryland, USA). The staining intensity was measured and expressed in arbitrary units standardised from 0 to 250, 0 being the maximum of the staining and 250 no staining. Ten fields for each section, selected at random, were examined and two types of measurements were performed for each placental component and for each field: a measurement of intensity only of the components reactive and a measurement of intensity of all the components (reactive and not reactive) observed in the field. Statistical analysis was performed using the *t* test for paired samples ($P < 0.05$ was considered significant). Then the following were tested: (1) differences between the data obtained from the first type of measurement of the control group and those of the other groups; (2) differences between the data obtained from the first and the second type of measurement in each study group. All the sections were examined by a single observer blinded to the origin of the organ under study. The reproducibility of the measuring analysis was assessed by comparing the measurements made by one observer at different times, and the measurements of two observers. Intra-observer and inter-observer coefficients of variation were 1.5% and 5.2%, respectively.

RESULTS

The clinical details of the women whose placentas were used for this study are reported in Table 1. Mean weeks of gestation at delivery was significantly lower in the cases with pre-eclampsia and with pre-eclampsia with HELLP

Table 2. Assessment of VEFG immunostaining in the placenta.

Study groups	Trophoblast	Vessels	Stromal cells	Hofbauer cells
Control	+	+	+	+
Hypertension	+	+	+	+
Pre-eclampsia	+*	+	+	+
Pre-eclampsia/HELLP syndrome	+*	+**	+§	+

+ = Reactivity; * = Reactivity in some tracts of trophoblast; ** = Reactivity in some vessels; § = Reactivity in cell clusters.

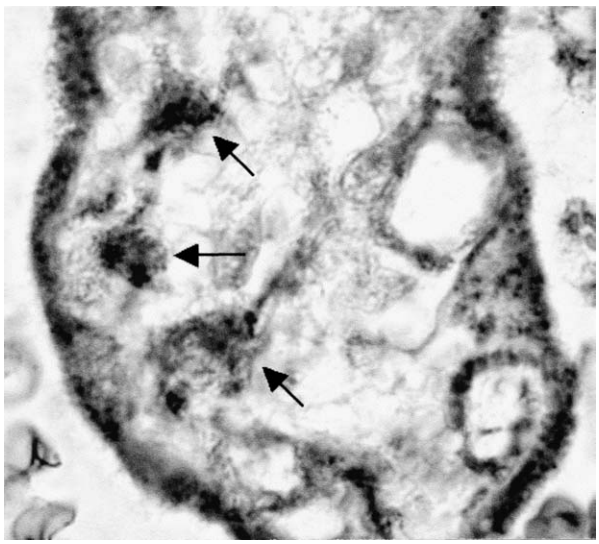

Fig. 1. VEGF immunostaining: Control, 38 weeks. The trophoblast, the endothelial cells of the vessels, the stromal cells and the Hofbauer cells (arrows) show reactivity. Original magnification ×1000.

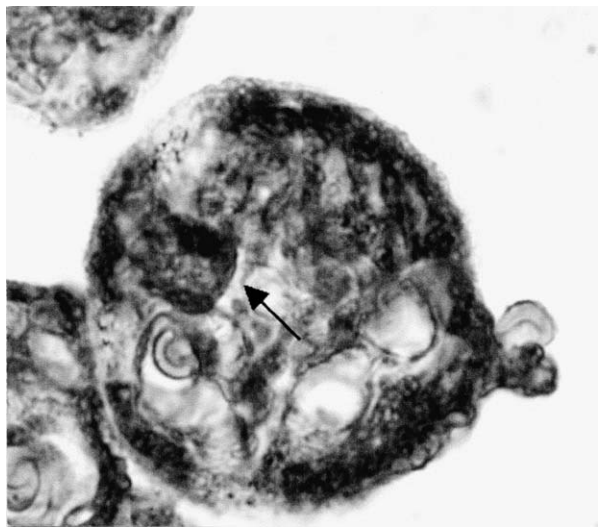

Fig. 3. VEGF immunostaining: Pre-eclampsia, 36 weeks. Some tracts of trophoblast, the endothelial cells of the vessels, the stromal cells and one Hofbauer cell (arrow) show reactivity. Original magnification ×1000.

syndrome ($P < 0.005$) with respect to the other cases. Mean birthweight ($P < 0.005$) and placenta weight ($P < 0.05$) were significantly lower in the cases with pre-eclampsia and pre-eclampsia with HELLP syndrome with respect to the others. Mean pressure (systolic and diastolic) was statistically higher ($P < 0.005$) in the cases with pre-eclampsia when compared with the cases with hypertension and with pre-eclampsia with HELLP syndrome; these last two cases showed mean values significantly higher ($P < 0.005$) with respect to the cases of control. The other clinical data did not show significant differences between study groups.

The study was limited to the chorionic intermediate and terminal villi (Table 2).

Control group: The trophoblast, the endothelial cells of the vessels, the stromal cells and the Hofbauer cells showed immunoreactivity (Fig. 1).

Hypertensive disorders groups: In the cases of gestational hypertension (Fig. 2) and of pre-eclampsia (Fig. 3), the various placental components were immunoreactive, although in the pre-eclampsia some tracts of trophoblast were not reactive.

In the cases of pre-eclampsia with HELLP syndrome, the trophoblast of the placenta showed reactivity, but only in some tracts. The endothelial cells of some vessels were reactive, while those of other vessels showed no reactivity.

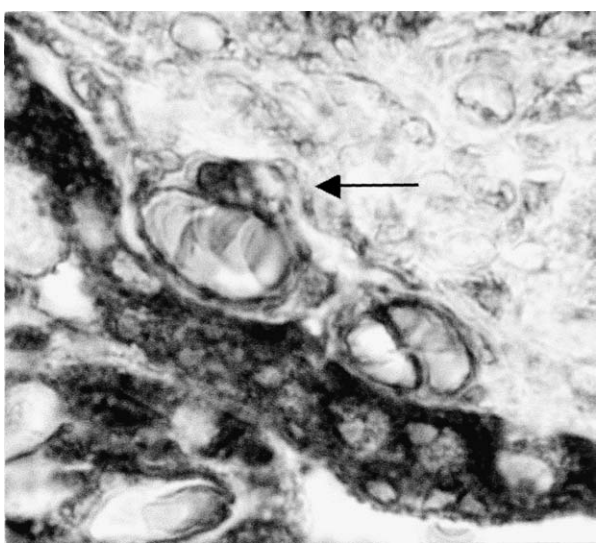

Fig. 2. VEGF immunostaining: Hypertension, 37 weeks. Reactivity is observed in the trophoblast, the stromal cells, the endothelial cells of the vessels and in one Hofbauer cell (arrow). Original magnification ×1000.

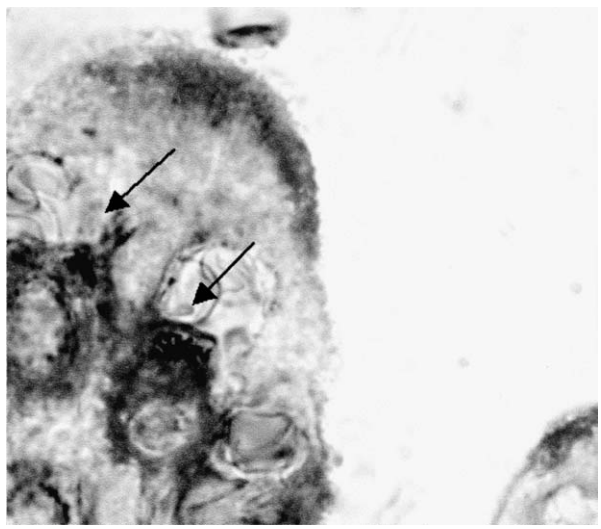

Fig. 4. VEGF immunostaining: Pre-eclampsia/HELLP syndrome, 36 weeks. Few tracts of trophoblast and the endothelial cells of some vessels are reactive. Two Hofbauer cells (arrows) show also reactivity. Original magnification ×1000.

Table 3. Values of mRNA concentration in the placenta. Values shown are mean (SEM).

Study groups	VEGF mRNA molecules/μg total RNA
Control	4.31×10^7 (3.36×10^6)
Hypertension	3.29×10^8 (3.71×10^7)*
Pre-eclampsia	4.61×10^7 (3.01×10^6)
Pre-eclampsia/HELLP syndrome	1.99×10^7 (2.33×10^6)**

* Hypertension *versus* control: $P < 0.0005$.
** Pre-eclampsia/HELLP syndrome *versus* control: $P < 0.005$.

In the stroma cell clusters and the Hofbauer cells were reactive (Fig. 4).

The statistical analysis with regard to the first type of measurement showed that the intensity of immunoreactivity of the trophoblast was higher in the cases of hypertension ($P < 0.001$), of pre-eclampsia ($P < 0.05$) and of pre-eclampsia with HELLP syndrome ($P < 0.005$) with respect to the control group. The intensity of reactivity of the vessels was significantly higher in the cases of hypertension ($P < 0.05$) and of pre-eclampsia with HELLP syndrome ($P < 0.05$) when compared with the control group; the vessels of the cases of pre-eclampsia did not show significant differences in reactivity with respect to those of the control group. The reactivity of the stromal cells was significantly higher in all the pathological cases (hypertension and pre-eclampsia with HELLP syndrome: $P < 0.001$; pre-eclampsia: $P < 0.01$). The Hofbauer cells showed a reactivity significantly higher in the cases of pre-eclampsia with HELLP syndrome when compared with the control group ($P < 0.01$). The Hofbauer cells of the other pathological cases did not show significant differences in reactivity with respect to the control group. The data obtained from the first type of measurement did not show significant differences when compared with those of the second type of measurement in the control group and in the cases of hypertension. In the cases of pre-eclampsia, the data of the second measurement on the trophoblast were significantly lower with respect to those of the first measurement ($P < 0.001$), while the data of the two measurements on the other placental components did not show significant differences. In the cases of pre-eclampsia with HELLP syndrome, the data of the second measurement on the trophoblast ($P < 0.001$), on the vessels ($P < 0.05$) and on the stromal cells ($P < 0.001$) were significantly lower with respect to those of the first measurement; the data of the two measurements on the Hofbauer cells did not show significant differences.

No statistically significant difference was revealed on the placental mRNA levels of VEGF in the cases of pre-eclampsia when compared with the mRNA levels in the control group ($P = 0.4$). In the cases of pre-eclampsia with HELLP syndrome the levels were statistically lower ($P < 0.005$) and in those of hypertension were higher ($P < 0.0005$) with respect to the control ones (Table 3).

DISCUSSION

This is the first study that investigates the expression of VEGF in the placenta from pregnancies complicated by hypertension disorders with various degrees of clinical severity. The data obtained in these cases were compared with those obtained in a group of physiological pregnancies considered as control group.

With regards the control group, our immunohistochemical results showed that VEGF was expressed in various placental components as observed in previous studies.[17,26,35,40] Moreover, in our study, VEGF immunoreactivity was also detected in the endothelial cells of the vessels that have not been observed previously. It is to be noted that in a study *in vitro* on the endothelial cells, an autocrine secretion of VEGF by these cells has been observed.[41] Therefore, VEGF immunoreactivity, which was present in the endothelial cells of the vessels, might be due to both the binding to its receptors and the production of this factor by these cells.

Our data on the determination of the presence of VEGF mRNA in the control group confirm the results of previous investigations, although the levels are not comparable because of the different assays employed.[8,18,36–38,42]

In the hypertension disorders groups, the location of VEGF in the placenta was the same with respect to the control group. In the cases of pre-eclampsia and, in particular, in the cases of pre-eclampsia with HELLP syndrome, some components did not express this factor, as demonstrated by the simple morphological observation and by the comparison of the data of the two types of measurements in each study group. However, the intensity of immunorectivity of the VEGF positive components was significantly higher in all the pathological groups with respect to the control one.

The VEGF mRNA levels in the cases of hypertension showed a statistically significant increase with respect to those of the control group. However, in pre-eclampsia, the levels of VEGF mRNA were not statistically different when compared with those of the control group, while in the pre-eclampsia with HELLP syndrome the levels were statistically lower.

These findings might be related to the different degrees of clinical severity of the various hypertensive disorders. It is known that in placentas from pregnancies with hypertension disorders, a hypoperfusion occurs followed by a hypoxic environment that stimulates the VEGF production.[3–6,12,15,16] Therefore, in the placenta of pregnancies with gestational hypertension, that are the lesser severe clinical cases, an increase of placental VEGF might be a compensatory mechanism in attempting to restore the blood flow toward normal. In the pregnancies with pre-eclampsia and with pre-eclampsia with HELLP syndrome, that according to some authors is a complication of the pre-eclampsia but in the opinion of others it can be also a separate entity,[43–48] there is also probably an attempt of

compensatory mechanism, but only some placental components seem be able to produce VEGF, as shown by immunohistochemistry. In some studies, it has been seen that VEGF, increasing in production with other factors, might have led to morphological and physiological alterations of the placental tissue that characterise the pre-eclampsia.[29,30,32,49–53] Therefore, in pre-eclampsia and in particular in pre-eclampsia with HELLP syndrome, there is probably an overproduction of VEGF in a definite period of gestation that might have led to the alteration and damage of the placental tissue, and then only some components might continue to overproduce VEGF. This fact might account for the lower levels of VEGF mRNA detected in these cases with respect to those in the cases of hypertension.

In previous studies on the expression of VEGF in placentas from pregnancies complicated by pre-eclampsia, a discrepancy of data was observed.[26,31,36] Our findings are partially in agreement with those of some of these investigations.[31,35,37,48] These disagreements might be due to an assemblage in one study group of cases of hypertensive disorders with different degrees of clinical severity and/or with a wide gestational age range and/or to an incorrect sampling of the tissue by the various authors. In our study, we attempted to form homogenous study groups of hypertensive disorders with a limited range of gestational age and we made a sampling random of the placental tissue in order to have a representative material of the whole organ for each case.

In conclusion, our data demonstrated that placental tissue from pregnancies with hypertensive disorders showed a different expression of VEGF according to different degrees of clinical severity.

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