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Comparative Study of Chorionic Villi Protein Expression between Normotensive Pregnancy and Preeclampsia

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Abstract

Objective: The study was undertaken to compare the chorionic villi protein expressions of vascular endothelial growth factor $(VEGF)_{165}$, $VEGF_{165}b$ and matrix metalloproteinase (MMP)-9 in preeclampsia and in normotensive pregnancies.

Methods: We studied 77 placentas, 53 from term deliveries of healthy women with uncomplicated pregnancies and 24 from women with preeclampsia. Protein expressions were determined by enzyme linked immunoassays (ELISA) using commercially available kits. The capture antibody for each of the ELISA kits was monoclonal antibody to the respective three human proteins.

Results: Race/ethnicity in the study was self -reported and the distribution showed that 65 % of the study population was of Hispanic and 35 % was of African American descent. Mann Whitney U test results show that chorionic villi $VEGF_{165}b$ protein expression differed significantly between normal pregnancies and preeclampsia (p = 0.0001). Spearman's rank correlation showed a significant positive correlation between the two isoforms of $VEGF_{165}$ (rho = +0.365, p = 0.007) in the third trimester of normal pregnancy, suggesting a synergistic action between the two proteins. In preeclampsia, this apparent synergy between the two VEGF proteins was not seen. In normal term delivery group, Spearman' s rank correlation showed $VEGF_{165}b$ protein expression was significantly and negatively correlated with MMP-9 (rho = -0.271, p = 0.049). In preeclampsia this correlation was not seen. In the normotensive group, $VEGF_{165}b$ protein expression in chorionic villi were found to be higher in women carrying a female fetus (p = 0.035); and placental weight in women carrying female fetuses was significantly higher (p = 0.032) as well. All studied variables were comparable between African American and Hispanic population except for $VEGF_{165}b$ which was significantly higher among African American population (p = 0.022). The systolic and diastolic blood pressure among African American population were also significantly higher (p = 0.016, 0.001, respectively).

Conclusions: The study underscores an important association between $VEGF_{165}b$ isoform and MMP-9 in normotensive pregnancy. Our findings strengthen our hypothesis that $VEGF_{165}b$ may play a significant role in normal human pregnancy. Our study corroborates previous reports that found African Americans to be at a higher risk for preeclampsia. In preeclampsia, vasoconstriction may be responsible for the elevated expression of $VEGF_{165}b$ protein, as tissue ischemia has been reported to stimulate $VEGF_{165}b$ levels. In the absence of a suitable animal model to study preeclampsia, more correlative data may contribute to a better understanding of the pathophysiology of preeclampsia in the future.

Keywords: *Vascular Endothelial Growth Factor (VEGF)165; Vascular Endothelial Growth Factor (VEGF)165b; Matrix Metalloproteinase (MMP)-9; ELISA; Protein Expression; Third Trimester Human Pregnancy*

Introduction

Placental angiogenesis plays a pivotal role in instituting a fetomaternal circulation and in establishing the placental villous tree, that contributes to the development of the placenta, throughout human pregnancy. The signals that initiate angiogenic growth of placental vascular network are multiple and complex. The search for potential regulators has yielded a number of growth factors e.g., five members of VEGF family, four members of the angiopoietin family and one member of large ephrin family, acidic and basic fibroblast growth factors, transforming growth factor -α, transforming growth factor-β, etc. [1-3]. These molecules were able to promote angiogenesis in certain model systems; however, the correlation of such activity with physiological or pathological regulation of blood vessel growth was difficult to ascertain [4]. Vascular endothelial growth factor (VEGF) on the other hand, was revealed to be specific for blood vessel formation [5]. Using gene knock-out mice, investigators have shown that when genes for VEGF receptors are specifically mutated, the mice demonstrate failure in hematopoiesis, failure in the formation of blood islands and blood vessels; and the embryos die by day 8 of mouse pregnancy [6]. Carmeliet and his group further demonstrated that the loss of a single VEGF allele in mice could lead to gross developmental deformities in vessel formation and the embryos die between day 11 and 12 of mouse pregnancy [7]. The authors concluded that fetal and placental angiogenesis were dependent on VEGF and that a threshold level of VEGF was absolutely essential, for normal vascular development to occur [7].

In early pregnancy, the VEGF protein is expressed in cytotrophoblasts at the implantation site and the expression of VEGF protein continues until term [8]. The decline in the post -delivery expression of VEGF suggests that placenta is the source of VEGF [9]. In previous reports VEGF A was referred to as VEGF [10]. Alternate splicing of VEGF A produces 6 mature proteins that vary in length from 121 - 206 amino acids. Among these, $VEGF_{165}$ is the dominant isoform identified in most tissues expressing VEGF gene [10].

In the past decade, the complexity of VEGF biology has intensified further. VEGF A gene which by alternate splicing yields five isoforms: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, were later discovered to yield additional isoforms based on alternate splicing of its exon 8. Even though these sister isoforms bears similar number of amino acids in their respective proteins, but the splicing event of exon 8 modifies the C-terminal end of the sister isoforms [10,11]. The former isoforms referred to as VEGF xxx are differentiated from their sister isoforms being referred to as VEGF xxxb. Following the splicing event of exon 8, the six former amino acids (CDKPRR) at the C-terminal end become replaced with six new amino acids (SLTRKD). This modification alters the tertiary structure of the protein, its overall charge, as well as the functional capacity of the sister protein s [12].

Growth of the placental villous tree depends also on how the decidual extracellular matrix is remodeled. The uterine stroma in early pregnancy undergoes a complex set of changes known as decidual reaction. Decidualization leads to striking changes in the size and shape of stromal uterine cells, accompanied by changes in their function [13]. Remodeling of this newly formed matrix is pivotal for the development of both the fetus and the placenta. Matrix metalloproteinases (MMPs), a family of matrix degrading enzymes participates in the remodeling process. The MMP family consists of 28 members and is categorized into different groups e.g. collagenases (MMP -1, MMP-8 and MMP-13); stromelysins (MMP -3, MMP-7, MMP-10) and gelatinases (MMP-2 and MMP-9) depending on the type of substrates the enzymes degrade [14]. In an *in vitro* study it was shown that activation of protein expression of MMP-9 is required for the invasion process [15].

Hemodynamic changes in maternal circulation occur during human pregnancy that increases the heart rate, plasma volume and cardiac output while maintaining the blood pressure of the woman at a steady state. This feat is primarily achieved by decreasing vascular resistance of the blood vessels. To meet the metabolic demands of the growing fetus placental blood volume progressively increases with an increase in gestational age. To accommodate greater volume of blood to pass through with minimal change in blood pressure, the spiral arteries are modified instead. The spiral arteries which were scrawny structures in non- pregnant state, during pregnancy are transformed into large flaccid blood vessels. Endovascular invasion of the spiral arteries by cytotrophoblasts replaces the endothelial and

smooth muscle cells that normally line these vessels. Since cytotrophoblasts lack vasoconstrictor capabilities the spiral arteries become transformed into flaccid blood vessels with decreased vascular resistance. This new transformation of the spiral arteries allows greater volume of blood to pass through without alteration in blood pressure. Failure to adequately remodel the spiral arteries during pregnancy, leads to placental ischemia, which is considered to be the initiating event that leads to preeclampsia [16].

The association between placental tissue and preeclampsia has been recognized for many decades [17]. Abnormal placentation has further been attributed as a contributing factor in preeclampsia, which has its origin in early pregnancy [18]. Trophoblasts, which are epithelia cells of ectodermal origin, during pregnancy acquire vascular like properties [19,20]. The cytotrophoblasts invade the maternal spiral arteries and replace the endothelial and smooth muscle cells that line these blood vessels. Replacement of the endothelial cells by cytotrophoblasts is under stringent spatial and temporal control and is limited to the endometrium and proximal myometrium and is terminated by midgestation [14,21]. In preeclampsia, however, the endovascular invasion by trophoblast is shallow that fails to undergo the complete spectrum of physiologic changes; furthermore, the invasion process does not proceed beyond the decidual portion of the spiral arteries. The mean external diameters of the myometrial spiral arteries in preeclampsia remain half the size of similar blood vessels from normal pregnancies [22]. Incomplete transformation of the spiral arteries triggers the release of pathologic factors and their inhibitors into the maternal circulation which result in maternal symptoms of preeclampsia [22,23]. The present study was undertaken to compare the placental protein expression of $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 between normotensive and preeclampsia to determine whether placental ischemia manifests an alteration in the expression patterns of any of the three studied proteins.

Methods

The investigative protocol for the study was approved by the Human Subject Ethics Committee of the BronxCare Health System, New York, USA. Normal women were normotensive throughout pregnancy and had no proteinuria or other signs of preeclampsia. The placentas were obtained when the women underwent cesarean sections between 37 to 40 weeks for obstetrical indications, as well from normal pregnancies delivered vaginally at 37 - 40 weeks of gestation. Preeclampsia patients fulfilled the following criteria, recommended by ACOG: no history of hypertension before pregnancy; onset of hypertension during late pregnancy with systolic and diastolic pressure > 140/90 mm Hg on at least two occasions and urinary protein >2+ on dipstick or >0.3 g/24 hr. In our study we had attempted to collect placentas from women with preeclampsia with gestational age of 36 weeks and above to keep the normotensive and preeclampsia group comparable. Women in the preeclampsia group did not have any previous history of preeclampsia. Pregnancies that were complicated with diabetes, hypertension, chronic renal disease and chronic peripheral vascular disease or with major fetal anomalies were not included in either of the study groups.

Placental tissue samples were collected within approximately 30 minutes of the procedures. Soon after collection, the placental tissues were processed to obtain chorionic villi samples. Briefly, portions of each placenta were first thoroughly washed in cold saline to remove maternal blood and were then dissected in saline to collect free floating chorionic villi that were not anchored to the basal plate nor were emerging from the chorionic plate surface vessels. Samples of floating chorionic villi, all from the same placenta were placed in separate tubes, each tube bearing the same ID# designated for that placenta. The sample collection tubes were then transported to the laboratory on ice and stored at -80^0C until assay.

The approved protocol allowed the collection of the following clinical information regarding the women from whom placental tissues were obtained. These included: maternal age, race/ethnicity (self - reported), gestational age (as determined by ultrasound or by initial date of the last menstrual period) and medicine(s) administered to induce termination of pregnancy. Tissues and clinical information were de-identified before exiting the delivery suites.

Chorionic villi $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 protein expressions were simultaneously determined by sandwich enzyme-linked immunoassay (ELISA) methods. The capture antibodies for the ELISA kits were monoclonal antibodies to human proteins $VEGF_{165}$ (DY293B), $VEGF_{165}b$ (DY3045) and MMP-9 (DY 911), respectively (R and D Systems, Minneapolis, MN). Chorionic villi samples were homogenized and supernatants following centrifugation of the homogenate at 13000 rpm for 2 minutes were used to determine protein expression based on the manufacturer's protocols. Each sample was assayed twice and the mean value of both measurements was used. Simultaneous analysis of both $VEGF_{165}$ and $VEGF_{165}b$ isoforms and MMP-9 meant that we carried out the protein's assays using chorionic villi tissues samples isolated from the same placentas but the assays were not carried out using the same aliquots. A Tecan infinite 200 Pro microplate reader (Tecan Systems Inc., San Jose, CA) set at 450 nm with wavelength correction set at 540 nm was used to measure absorbance. The sensitivity of the ELISA assays for $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 were 31.3 pg/ml, 62.5 pg/ml and 31.3 pg/ml, respectively. Intra -assay and inter -assay variations for the assays were between 7 - 10%.

Statistical analysis

The statistical software package SPSS, version 25 (IBM Corporation, Armonk, NY) was used for statistical analyses. The normality of the data when analyzed statistically showed that the data was not normally distributed. Hence, non -parametric statistics were applied. The data was grouped by trimesters and the following non-parametric tests were performed: 1) Mann Whitney U test was applied to compare the normotensive and the preeclamptic groups; and 2) Spearman Rank correlation coefficient test was applied to summarize the strength and direction of a relationship between the variables as well as between thetwo study groups. A correlation was considered significant for 2 -tailed p-value < 0.05.

Results and Discussion

In the present study, chorionic villi protein expression of $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 were compared between normotensive and preeclamptic placentas. A total of 77 placental samples were analyzed, 24 placentas were from women with preeclampsia and 53 were from the normotensive group. Table 1 shows the clinical characteristics of the women from whom placentas were taken. The average maternal age of the two groups was comparable. The average gestational age of the normotensive group was significantly higher ($p = 0.013$). Systolic and diastolic blood pressure of women with preeclampsia were both significantly higher ($p = 0.0001$). Placental weight of the two groups was comparable. In the preeclampsia group, the newborns weights were significantly lower ($p = 0.0001$). Distribution of self -reported race/ethnicity showed that 73% of the normotensive group was of Hispanic origin and 27% were African Americans. In the preeclampsia group, 46% were Hispanic and 54% were African Americans. A total of 39 women in the normotensive group delivered by C-section and 14 women delivered vaginally. In the preeclampsia group, 9 women delivered by C-section and 15 women delivered vaginally. Information on the gender of newborns showed that in the normotensive group 21 newborns were females and 25 males, in the preeclampsia group, 10 newborns delivered were females and 12 were males. Information on the gender of 9 newborns was missing, 7 in the normotensive group and 2 in the preeclampsia group.

During pregnancy, vasculogenesis and angiogenesis are required in the fetal compartment. The maternal compartment requires angiogenesis and placental angiogenesis is controlled by angiogenic factors. Gestational studies have confirmed that growth of the villi and capillaries are biphasic with slower growth occurring before mid -gestation and rapid growth thereafter [24]. An important aspect of placental angiogenesis is that cytotrophoblasts involved in the angiogenic process of early pregnancy differ significantly from the ones that are involved in angiogenesis in the later part. In early pregnancy, the cytotrophoblasts have specific sets of genes and cell surface receptors that are capable of responding to environmental or paracrine stimuli. These cytotrophoblasts participate in angiogenesis before the onset of any circulation or before circulation is functionally necessary [8]. Signals to form new blood vessels depend on factors that are released from tissues that need to be vascularized. Placental angiogenesis in the second and third trimester s of human pregnancy are regulated by different sets of genes that respond to the physiological requirement of the tissue, fulfilling the increase in metabolic demands, as the fetus develops [8].

Table 1: *Characteristics of women from whom placentas were taken.*

Groups	Normotensive Pregnancy	Preeclampsia	p value
N	53	24	
Age (years)	27.5 ± 6.2	26.2 ± 5.9	0.393
Gestational Age in Days	276 ± 6.1	258 ± 28.7	0.005
Systolic Blood Pressure (mm Hg)	123 ± 18	154 ± 18	0.0001
Diastolic Blood Pressure (mm Hg)	72 ± 13	92 ± 14	0.001
Placental weights (gm s)	453 ± 107	41 ± 149	0.220
Newborn weights (gms)	3365 ± 499	2630 ± 899	0.0001
Ethnicity			
Black	14	13	
Hispanic	39	11	
Method of Delivery			
C-Section	39	9	
Vaginal	14	15	
Gender of Newborn			
Female	21	10	
Male	25	12	
Missing data	7	2	

Among the various isoforms of VEGF, the 165 amino acid form is most abundant *in vivo* [25]. In earlier reports $VEGF_{165}$ was recognized as VEGF. Involvement of $VEGF_{165}$ in human pregnancy, particularly in placental development has been recognized. In earlier reports, placental expression of $VEGF_{165}$ protein was stated to increase in first 10 weeks of normal pregnancy but as pregnancy advanced, $VEGF_{165}$ and its receptor VEGFR-2 concentrations were reported to decline [4,5]. Compared to $VEGF_{165}$, the $VEGF_{165}b$ isoform has not been investigated in as much details.

In the present study, the three studied proteins were identified in all chorionic villi samples that were analyzed by ELISA. The capture antibodies used for the assays were monoclonal antibodies to their respective proteins. Mann Whitney U test applied to examine the difference in chorionic villi protein expressions between the two groups revealed that while $VEGF_{165}$ and MMP-9 expressions were comparable between the two groups, chorionic villi $VEGF_{165}b$ protein expression was significantly higher in women with preeclampsia ($p = 0.001$) (Table 2). Graphical representation of the expression patterns of the three proteins are shown in figure 1 and 2. The comparable levels of $VEGF_{165}$ seen in this study between preeclampsia and normal placentas is consistent with a report published earlier [26]. The significantly higher $VEGF_{165}b$ protein expression in preeclamptic placentas seen in the present study (Table 2, Figure 1 and 2) is in contrast with a published report that showed lower levels of $VEGF_{165}b$ protein in preeclamptic placentas compared to normal third trimester group [10].

In a previous study, we had identified both forms of $VEGF_{165}$ in chorionic villi placental samples throughout gestation in normal pregnancy; and a gestational age -specific difference in the expression of the two $VEGF_{165}$ isoforms were noted [27]. The results showed that the ratio of the two $VEGF_{165}$ isoforms differed significantly between trimesters. In the first trimester the ratio of $VEGF_{165}b$ to $VEGF_{165}$ isoform was 1.9, in the second trimester the ratio of $VEGF_{165}b$ to $VEGF_{165}$ isoform increased to 6.3; and in the third trimester, the ratio dropped from the second trimester value to a value of 2.3 [27]. The ratio of $VEGF_{165}b$ to $VEGF_{165}$ in the present study in the normotensive group, i.e. in the third trimester placentas is 2.5, consistent with our previous report [27]. In the preeclampsia group (also third trimester) in the present study, the ratio of $VEGF_{165}b$ to $VEGF_{165}$ is 4.9, a value twofold higher than that of normal third trimester.

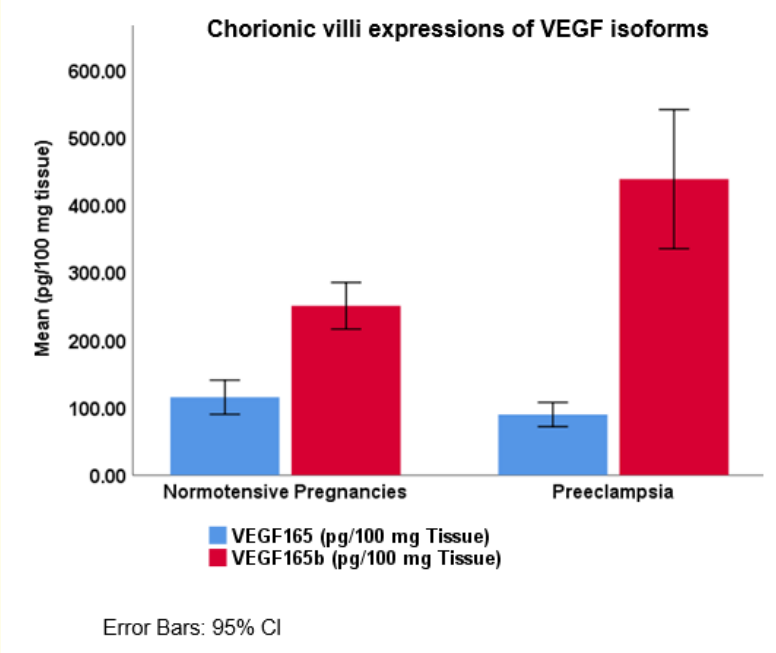

Figure 1

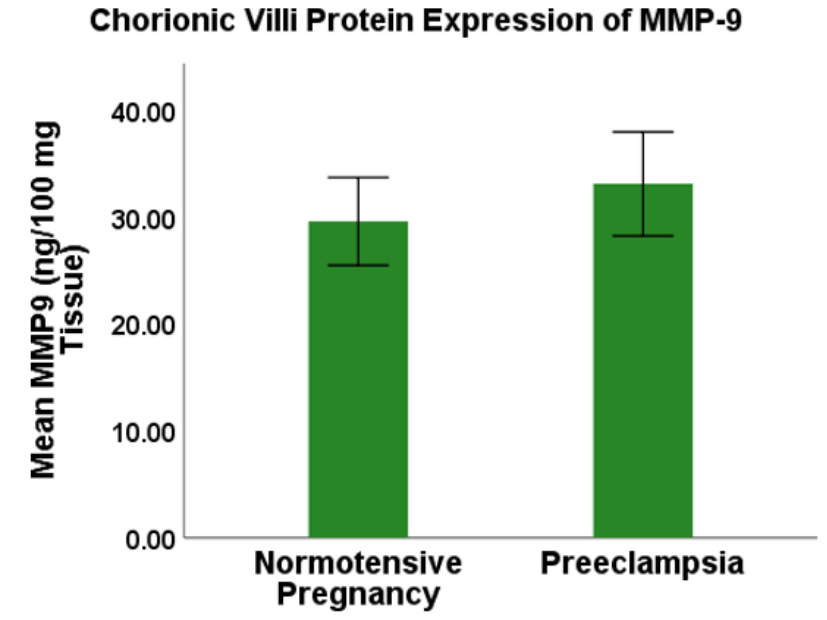

Figure 2

Table 2: *Chorionic villi Protein Expressions.*

Groups	Normotensive Pregnancy	Preeclampsia
	Median (N = 53)	**Median (N = 24)**
$VEGF_{165}$ (pg/100 mg tissue)	90.05 (52.65 - 278.00)	81.60 (56.65 - 161.85)
$VEGF_{165}b$ (pg/100 mg tissue)	223.84 (87.09 - 398.75)	402.75 (95.70 - 668.20)
MMP-9 (ng/100 mg tissue)	26.12 (20.87 - 48.97)	34.74 (22.92 - 49.08)

$VEGF_{165}$: Vascular Endothelial Growth Factor$_{165}$; $VEGF_{165}b$: Vascular Endothelial Growth Factor$_{165}$b; MMP-9: Matrix Metalloproteinase-9. Homogenized human placental chorionic villi samples were analyzed using ELISA assay kit s from R&D Systems, Minneapolis, MN to determine $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 protein expressions. Data were not normally distributed, hence, non-parametric statistics were used; and the median and the range for each protein are shown in the table.

Correlations between the studied variables were determined individually for the normal term delivery and the preeclampsia group using Spearman's Rank correlation. Results as shown in table 3 indicate that in the normotensive group, $VEGF_{165}b$ was significantly and positively correlated with $VEGF_{165}$ (rho = +0.365, p = 0.007). Similar significant positive correlations between the two $VEGF_{165}$ isoforms in the third trimester of normal pregnancy were reported earlier [10,27]. In the preeclampsia group in the present study, this correlation was not seen (Table 4). Fetal development and the metabolic demands of the fetus both attain their peaks in third trimester of human pregnancy. The significant positive correlation between the two isoforms of $VEGF_{165}$, as seen in the normotensive group in the present study (Table 3) suggests that a synergistic action of both isoforms of $VEGF_{165}$ is perhaps necessary for placental vascular modification that would allow maximum blood to flow through, with least vascular resistance. In preeclamptic placentas, the synergy between the two VEGF proteins was absent (Table 4).

Table 3: *Correlation between studied variables in normotensive pregnancies. Spearman Rank Correlation Results.*

Variables		$VEGF_{165}$	$VEGF_{165}b$	MMP-9	Age	GAD	Sys Pr	Dia Pr	Pl Wt	NB Wt
$VEGF_{165}$ (pg/100 mg tissue)	Corr Coeff	1.000	0.365	-0.228	0.161	-0.158	-0.163	-0.089	0.163	0.071
	Sig (2-Tailed)		**0.007**	0.100	0.251	0.259	0.263	0.544	0.242	0.631
	N	53	53	53	53	53	49	49	53	48
$VEGF_{165}b$ (pg/100 mg tissue)	Corr Coeff	0.365	1.000	-0.271	0.039	-0.095	0.187	0.383	-0.079	0.012
	Sig (2-Tailed)	**0.007**		**0.049**	0.780	0.499	0.199	0.007	0.576	0.936
	N	53	53	53	53	53	49	49	53	48
MMP-9 (ng/100 mg tissue)	Corr Coeff	-0.228	-0.271	1.000	-0.054	-0.038	-0.147	-0.200	0.084	-0.151
	Sig (2-Tailed)	0.100	**0.049**		0.703	0.786	0.313	0.168	0.549	0.307
	N	53	53	53	53	53	49	49	53	48
Age (years)	Corr Coeff	0.161	0.039	-0.054	1.000	-0.142	0.008	-0.005	0.120	0.160
	Sig (2-Tailed)	0.251	0.780	0.703		0.310	0.955	0.974	0.391	0.278
	N	53	53	53	53	53	49	49	53	48
Gestational age in days	Corr Coeff	-0.158	-.0095	- 0.038	-0.142	1.000	0.033	0.141	-0.085	.347
	Sig (2-Tailed)	0.259	0.499	0.786	0.310		0.822	0.335	0.546	**0.016**
	N	53	53	53	53	53	49	49	53	48
Systolic Blood Pressure (mm Hg)	Corr Coeff	-0.163	0.187	-0.147	0.008	0.033	1.000	.722	-0.137	0.203
	Sig (2-Tailed)	0.263	0.199	0.313	0.955	0.822		**0.0001**	0.349	0.172
	N	49	49	49	49	49	49	49	49	47
Diastolic Blood Pressure (mm Hg)	Corr Coeff	-0.089	0.383	- 0.200	-0.005	0.141				
	Sig (2-Tailed)	0.544	**0.007**	0.168	0.974	0.335	**0.0001**		0.357	0.422
	N	49	49	49	49	49	49	49	49	47
Placental Weight (gms)	Corr Coeff	0.163	-0.079	0.084	0.120	-0.085	-0.137	-0.134	1.000	0.583
	Sig (2-Tailed)	0.242	0.576	0.549	0.391	0.546	0.349	0.357		**0.0001**
	N	53	53	53	53	53	49	49	53	48
NewBorn Weight (gms)	Corr Coeff	0.071	0.012	- 0.151	0.160	0.347	0.203	0.120	0.583	1.000
	Sig (2-Tailed)	0.631	0.936	0.307	0.278	**0.016**	0.172	0.422	**0.0001**	
	N	48	48	48	48	48	47	47	48	48

$VEGF_{165}$: Vascular Endothelial Growth Factor$_{165}$; $VEGF_{165}b$: Vascular Endothelial Growth Factor$_{165}$b; MMP-9: Matrix Metalloproteinase-9; GAD: Gestational age in days; Sys Pr: Systolic Blood Pressure; Dia pr: Dastolic Blood Pressure; Pt Wt: Placental Weight; NB Wt: New Born Weight. Statistically significant results are highlighted.

Table 4: *Correlation between studied variables in preeclamptic pregnancies. Spearman Rank Correlation Results.*

Variables		$VEGF_{165}$	$VEGF_{165}b$	MMP-9	Age	GAD	Sys Pr	Dia Pr	Pl Wt	NB Wt
$VEGF_{165}$ (pg/ 100mg tissue)	Corr Coeff	1.000	0.377	0.121	0.231	-0.117	-0.343	-0.021	0.257	0.101
	Sig (2-Tailed)		0.070	0.574	0.277	0.586	0.101	0.923	0.225	0.639
	N	24	24	24	24	24	24	24	24	24
$VEGF_{165}b$ (pg/ 100mg tissue)	Corr Coeff	0.377	1.000	-0.290	0.128	-0.234	0.047	0.059	0.013	-0.197
	Sig (2-Tailed)	0.070		0.169	0.522	0.271	0.826	0.785	0.953	0.355
	N	24	24	24	24	24	24	24	24	24
MMP-9 (ng/ 100mg tissue)	Corr Coeff	0.121	-0.290	1.000	-0.025	0.088	0.033	0.223	0.308	0.173
	Sig (2- Tailed)	0.574	0.169		0.907	0.684	0.880	0.294	0.143	0.419
	N	24	24	24	24	24	24	24	24	24
Age (years)	Corr Coeff	0.231	0.128	-0.025	1.000	-0.197	-0.236	-0.218	-0.058	0.008
	Sig (2-Tailed)	0.277	0.522	0.907		0.357	0.268	0.305	0.786	0.971
	N	24	24	24	24	24	24	24	24	24
GAD	Corr Coeff	-0.117	-0.234	0.088	-0.197	1.000	-0.157	-0.317	0.570	0.771
	Sig (2- Tailed)	0.586	0.271	0.684	0.357		0.462	0.131	0.004	**0.0001**
	N	24	24	24	24	24	24	24	24	24
Systolic Blood Pressure (mm Hg)	Corr Coeff	-0.343	0.047	0.033	-0.236	-0.157	1.000	0.724	-0.028	-0.306
	Sig (2-Tailed)	0.101	0.826	0.880	0.268	0.462		**0.0001**	0.897	0.146
	N	24	24	24	24	24	24	24	24	24
Diastolic Blood Pressure (mm Hg)	Corr Coeff	-0.021	0.059	0.223	-0.218	-0.317	0.724	1.000	-0.123	-0.391
	Sig (2- Tailed)	0.923	0.785	0.279	0.30	0.131	**0.0001**		0.568	0.059
	N	24	24	24	24	24	24	24	24	24
Placental Weight (gms)	Corr Coeff	0.257	0.013	0.308	-0.058	0.570	-0.028	-0.123	1.000	0.689
	Sig (2-Tailed)	0.225	0.953	0.143	0.786	**0.004**	0.897	0.568		**0.0001**
	N	24	24	24	24	24	24	24	24	24
Newborn Weight (gms)	Corr Coeff	0.101	-0.197	0.173	0.008	0.771	-0.306	-0.391	0.669	1.000
	Sig (2- Tailed)	0.639	0.355	0.419	0.971	**0.0001**	0.146	0.059	**0.0001**	
	N	24	24	24	24	24	24	24	24	24

$VEGF_{165}$: Vascular Endothelial Growth Factor$_{165}$; $VEGF_{165}b$: Vascular Endothelial Growth Factor$_{165}$b; MMP-9: Matrix Metalloproteinase-9; GAD: Gestational Age in Days; Sys Pr: Systolic Blood Pressure; Dia pr: Diastolic Blood Pressure; Pt Wt: Placental Weight; NB Wt: New born Weight. Statistically significant results are highlighted.

Alternate splicing of exon 8 of VEGF A gene, as reported in previous studies, yields two different isoforms of $VEGF_{165}$; proximal splicing of exon 8 yields $VEGF_{165}$ and distal splicing yields $VEGF_{165}b$ [12,28]. Based on the results of our previous study we hypothesized that hypoxic environment in the first trimester of normal pregnancy favors proximal splicing of exon 8 and $VEGF_{165}$-induced angiogenesis. At the end of first trimester, when placental environment switches from hypoxic to a normoxic state, the distal splicing of exon 8 is favored,

whereby the expression of $VEGF_{165}b$ protein gets upregulated. In the third trimester of normal pregnancy the upregulation in expression of $VEGF_{165}b$ protein no longer prevails rather, a downregulation of $VEGF_{165}b$ protein expression occurs resulting in both isoforms of $VEGF_{165}$ being expressed in equal amounts [27]. In the present study, in preeclamptic placentas (third trimester) a significantly higher $VEGF_{165}b$ protein expression is seen compared to normal third trimester group (Table 2, Figure 1). We hypothesize that perhaps distal splicing of exon 8 was also favored in the second trimester of preeclamptic pregnancy where by the protein expression of $VEGF_{165}b$ became upregulated. However, since the mechanism that should have downregulated the expression of $VEGF_{165}b$ in the third trimester is perhaps lacking in preeclamptic placentas, the upregulation of $VEGF_{165}b$ protein expression persists. We hypothesize that this imbalance between the two VEGF isoforms in the preeclampsia group in the third trimester may negatively impact the angiogenic process. It may also be feasible that in preeclamptic state, failure in the endoinvasion of the spiral arteries by cytotrophoblasts leaves the spiral arteries unmodified, leading to ischemic blood vessels [21]. Vasoconstriction that results due to failure in the modification of the spiral arteries is perhaps responsible for the elevation in $VEGF_{165}b$ protein in preeclampsia, similar to that which happens in peripheral artery disease. Tissue ischemia which is generated through arterial occlusion and insufficient collateral vessel formation in peripheral artery disease, show a concomitant increase in the expression of $VEGF_{165}b$ protein as well [29].

Spearman rank correlation results as shown in table 3 indicate that in the normotensive pregnancy, $VEGF_{165}b$ was significantly but negatively correlated with MMP-9 (rho = -0.271, p = 0.049). In two separate previous studies we have shown: 1) placental expression of MMP -9 in normal pregnancy progressively increases with an increase in gestational age (30); and 2) $VEGF_{165}b$ protein expression is downregulated in the third trimester of normal pregnancy compared to second trimester levels [27]. In the current study, a significant negative correlation is noted between $VEGF_{165}b$ and MMP-9 protein expressions (rho = -0.271, p = 0.049, Table 3) in the third trimester of normal pregnancy. We hypothesize, that perhaps the downregulation of $VEGF_{165}b$ protein in the third trimester of normal pregnancy, is brought about by MMP-9, to restrain the angiogenic process from progressing any further. In the preeclampsia group in the present study, this negative association between $VEGF_{165}b$ and MMP-9 protein was not seen.

It is well known that newly formed capillaries in the placenta necessitate the progressive degradation of the decidual extracellular matrix by proteolytic enzymes [31,32]. The breakdown and reorganization of the decidual matrix is regulated by MMPs [31,32]. The involvement of MMP-9 in human pregnancy was accepted when it was found that MMP-9 is involved in neovascularization [33], the protein bears a significant positive correlation with gestational age in days in the first trimester of normal pregnancy [34] and an in vitro study showed that MMP-9 participates specifically in the degradation of ECM [15]. In the present study, MMP-9 protein expression was found not to differ between preeclamptic and control group. Our results are in agreement with a report in which immunostaining for MMP -9 in interstitial cytotrophoblasts was found to be comparable between preeclamptic and control specimens [35]; and is similar to a report that showed unchanged circulating MMP -9 levels between preeclamptic and normotensive pregnant women [36].

Spearman Rank correlation results shown in table 3 and 4 reveal that maternal systolic and diastolic blood pressure were significantly correlated in both normotensive pregnancies (rho = +0.722, p = 0.0001, Table 3) as well as in preeclampsia (rho = +0.383, p = 0.007, Table 4). Additionally, diastolic blood pressure in the normotensive group was found to be significantly correlated with $VEGF_{165}b$ protein expression (rho = +0.383, p = 0.007, Table 3). Gestational age in days was positively correlated with new born weights (rho = +0.347, p = 0.016) but not with placental weights in the normotensive group (Table 3); but with both placental and newborn weights (rho = +0.570, p = 0.004; rho = +0.771, p = 0.0001, respectively) in preeclampsia (Table 4). Positive significant correlations between placental and new born weights were seen both in the normotensive group (rho = +0.583, p = 0.0001, Table 3) as well as in the preeclampsia group (rho = +0.669, p = 0.0001) (Table 4). This positive correlation seen between placental and newborn weights in both normotensive and preeclampsia groups signify that the fetuses in utero were stronger and better equipped to bear the trauma of childbirth and was the reason why the mothers from whom placentas were collected for the study experienced positive pregnancy outcomes in the end.

Chorionic villi expression of the three proteins was not found to be affected by the method of delivery used. The data was additionally analyzed to determine the effects of ethnic variation on the studied variables. Race/ethnicity in the present study was self-reported. For the analysis both normotensive and preeclampsia groups were combined and the data was analyzed by independent T test. The findings as presented in table 5 show that the expression patterns of $VEGF_{165}$, MMP-9, age, gestational age in days, placental weight and newborn weight were all comparable between Black and Hispanic population. Chorionic villi expression of $VEGF_{165}$b protein was, however, significantly higher among African Americans (p = 0.022) and; among African Americans, both maternal systolic and diastolic blood pressure values were higher (p = 0.016, p = 0.001, respectively; Table 5). That African American women are at a higher risk of preeclampsia compared with Caucasians has been reported previously in a large study that surveyed hospital discharge reports from 1979 through 2006, that included 4644 African American and 12131 Caucasian women and was adjusted for a multitude of parameters [37]. A CDC report also states that the rate for preeclampsia for Black women is 61% higher than for White women and 50% higher than for women overall [38]. Our study corroborates these previous reports finding African Americans to be at a higher risk for preeclampsia.

Table 5: *Effects of ethnic variations on studied variables. Results of Independent T test.*

Variables	Ethnicity	N	Mean	Std. Deviation	Sig. 2-Tailed
VEGF165 (pg/ 100mg tissue)	Black	27	103.40	69.22	
	Hispanic	50	109.53	86.64	0.736
VEGF165b (pg/ 100mg tissue)	Black	27	386.80	234.18	
	Hispanic	50	267.68	149.55	0.022
MMP-9 (ng/ 100mg tissue)	Black	27	32.76	15.35	
	Hispanic	50	29.66	13.22	0.369
Age (years)	Black	27	26.59	7.02	
	Hispanic	50	27.38	6.09	0.625
Gestational Age (in days)	Black	27	267.89	18.6	
	Hispanic	50	271.50	18.8	0.422
Systolic Blood Pressure (mm Hg)	Black	25	141	23	
	Hispanic	48	128	21	**0.016**
Diastolic Blood Pressure (mm Hg)	Black	25	88	17	
	Hispanic	48	74	14	**0.001**
Placental Weight (gm)	Black	27	453.7	157.8	
	Hispanic	50	432.3	99.4	0.527
Newborn Weight (gm)	Black	24	3047.8	766.7	
	Hispanic	48	3165.8	726.8	0.566

$VEGF_{165}$: Vascular endothelial growth factor$_{165}$; $VEGF_{165}$b: Vascular endothelial growth factor$_{165}$b; MMP-9: Matrix metalloproteinase -9; GAD: Gestational age in days; Sys Pr: Systolic blood pressure; Dia pr: Dastolic blood pressure; Pt Wt: Placental weight; NB Wt: New bornweight. Statistically significant results are highlighted.

We additionally examined whether the gender of the fetus the mother was carrying had any effect on the studied variables. To eliminate any confounding effect of preeclampsia we restricted the analysis to the normotensive group. Interestingly we found a significant impact of fetal gender on maternal placental weight and on placental $VEGF_{165}$b protein expression. The data as shown in table 6 demonstrate that placental weight in women carrying female fetuses was significantly higher (p = 0.032); and chorionic villi expression of $VEGF_{165}$ protein in women carrying female fetuses was significantly higher as well (p = 0.035).

Table 6: *Effects of gender of the fetus the mother was carrying on the studied va riables. Results of Independent T test.*

Variables	Gender of Fetus	N	Mean	Std. Deviation	Sig. 2-Tailed
$VEGF_{165}$ (pg/ 100mg tissue)	Female	21	156.28	102.38	
	Male	25	88.90	74.65	0.017
$VEGF_{165}b$ (pg/ 100mg tissue)	Female	21	264.12	124.71	
	Male	25	250.95	132.24	0.730
MMP-9 (ng/ 100mg tissue)	Female	21	29.75	15.74	
	Male	25	26.85	9.68	0.468
Age (years)	Female	21	29.7	6.4	
	Male	25	26.8	6.5	0.131
Gestational Age (in days)	Female	21	277.6	6.1	
	Male	25	275.4	6.4	0.239
Systolic Blood Pressure (mm Hg)	Female	21	120	15	
	Male	25	125	18	0.314
Diastolic Blood Pressure (mm Hg)	Female	21	71	11	
	Male	25	73	15	0.57
Placental Weight (gms)	Female	21	492.9	85.8	
	Male	25	397.6	83.4	0.0001
Newborn Weight (gms)	Female	21	3465.8	452.8	
	Male	25	3264.0	540.0	0.175

$VEGF_{165}$: Vascular Endothelial Growth Factor$_{165}$; $VEGF_{165}b$: Vascular Endothelial Growth Factor$_{165}b$; MMP-9: Matrix Metalloproteinase-9; GAD: Gestational Age in Days; Sys Pr: Systolic Blood Pressure; Dia pr: Diastolic Blood Pressure; Pt Wt: Placental Weight; NB Wt: Newborn Weight. Statistically significant results are highlighted.

The limitation of our study is that it would have been better if we could confirm our data by western blot, polymerase chain reaction or by immunohistochemical methods. Moreover, it would have been helpful if we could in the present study include other forms of VEGF and MMP proteins that have been detected in human placenta. Furthermore, we realize that the activity of MMP-9 could have been influenced by other MMP-9 activators or inhibitors but changes in the expression of these factors have not been currently investigated. All members of MMP family are produced as secreted proenzymes. The activation of the enzymes proceeds in a stepwise fashion, in which parts of the enzyme are cut off until a fully activated form is achieved. The reason for this stepwise activation is because the MMPs are destructive enzymes and hence the production of their active forms is held in check by their stepwise processing; so that the activities of these enzymes could be controlled. The immunoassay that we have currently used, assays both the proactive and the active forms. We are now planning to investigate the pro-enzyme and the active form separately and then investigate the impact of MMP-9 on VEGF isoforms or on other placental parameters. The strength of our study is the relatively larger sample size in both normotensive and preeclampsia groups. Additionally, stringent ELISA methods that used monoclonal antibodies to human $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 antibodies as capture antibody were used. The ELISA methods used were sensitive, allowing the detection of $VEGF_{165}$, $VEGF_{165}b$ and MMP-9 as low as 31.2 pg/ml, 62.5 pg/ml and 31.2 pg/ml, respectively.

Conclusion

Our findings strengthen our hypothesis that $VEGF_{165}b$ plays a significant role in normal human pregnancy. The study underscores an important association between $VEGF_{165}b$ isoform and MMP-9 in normal human pregnancy. Our findings suggest a notable difference between tumor and placental angiogenesis. It is widely reported that in human tumors, up -regulation of $VEGF_{165}$ protein occurs with a proportional drop in $VEGF_{165}b$ levels; indicating that in tumor angiogenesis the balance between the two isoforms of VEGF is lost [39]. However, the findings of our current and previous studies suggest that gestational age -specific expression of both $VEGF_{165}$ isoforms is necessary for optimal placental angiogenesis and growth to results in a successful viable outcome.

It needs to be underscored that preeclampsia is recognized as a variable disease. In the absence of suitable animal model to study preeclampsia, more correlative data at different periods of human gestation may contribute to a better understanding of the pathophysiology of preeclampsia in the future. An increase in the information of tested biomarkers can only broaden our knowledge pertaining to the disease; and the biomarkers could in turn be used in designing newer focused studies on chorionic villi protein expression in normal and abnormal pregnancies.

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Conflict of Interest

The authors declare no conflicts of interest with respect to the research, authorship and/ or publication of this article.

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