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EC GYNAECOLOGY
Research Article

Can Chorionic Villi Angiogenic Protein Expression Clarify the Vascular Abnormality Seen in Preeclampsia?

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Received: August 04, 2022; **Published:** August 18, 2022

Abstract

Objective: We have investigated the expression patterns of chorionic villi vascular endothelial growth factor $(VEGF)_{165}$, $VEGF_{165}b$, matrix metalloproteinase (MMP)-9 and tumor necrosis factor alpha (TNF-α) proteins in normal pregnancies and preeclampsia to determine if the protein expression patterns could contribute in explaining the vascular abnormality seen in preeclampsia. Differences in the placental proteins between Black American and Hispanic groups were explored.

Methods: Term placentas from normotensive healthy women and placentas from women (37 weeks+ gestation) with preeclampsia, diagnosed by ACOG's criteria were investigated. Chorionic villi expressions of the four proteins were determined by ELISA; each ELISA kit using monoclonal antibody to the respective human proteins as capture antibody.

Results: Independent T test comparing normal pregnancy and preeclampsia showed placental $VEGF_{165}$ protein was higher in normal pregnancy ($p = 0.001$); $VEGF_{165}b$, MMP-9 and TNF-α proteins were higher in preeclampsia ($p = 0.05$, $p = 0.036$ and $p = 0.037$, respectively). Mothers' systolic and diastolic blood pressures were higher ($p = 0.000$), placental weight was comparable; and newborn weights were lower ($p = 0.000$) in preeclampsia. In normal pregnancy, the two VEGF165 isoforms were correlated ($r = 0.261$, $p = 0.005$) and $VEGF_{165}$ was negatively correlated to MMP-9 protein ($r = -0.185$, $p = 0.047$). Ethnicity was self-reported and the distribution was 23.3% of Black Americans and 69.8% of Hispanic American descent. Study variables differed between the ethnic groups.

Conclusion: Preeclampsia is a multifactorial disease. The findings suggest that placental angiogenic proteins may be contributory factors in the pathophysiology of the disease. Differences in $VEGF_{165}$ ratios, mothers' blood pressure and newborn weights were significantly different between normal pregnancy and preeclampsia, while placental weight was comparable, which suggests that placental angiogenic processes of the two groups may be different. The synergistic actions of $VEGF_{165}$ proteins in the third trimester of normal pregnancy, we suggest, may have favored sprouting angiogenesis maximizing blood flow; resulting in healthy newborns with normal weight. The imbalance in placental $VEGF_{165}$ proteins, we hypothesize, may be an angiogenic biomarker triggering angiogenesis of the intussusceptive type. The higher expression of MMP-9 protein in preeclampsia may facilitate intussusceptive angiogenesis to breakdown the extracellular matrix, to allow the capillary plexus to grow. Higher TNF-α protein in preeclampsia may be one of the compensatory mechanisms to assist the dilation of the blood vessels allowing greater amounts of blood to be transported to the fetus. The findings of this study additionally revealed that the correlations between the studied placental proteins differed significantly between the two ethnic groups.

Keywords: *Pregnancy-Specific Protein Expressions; Normal Human Pregnancy; Preeclampsia, $VEGF_{165}$, $VEGF_{165}b$, MMP-9 and TNF-α Proteins; Hispanic and Black Ethnic Groups; Ethnic Differences, Methods of Delivery, Fetal Gender*

Abbreviations

$VEGF_{165:}$ Vascular Endothelial Growth Factor $(VEGF)_{165}$; $VEGF_{165}b$: Vascular Endothelial Growth Factor $(VEGF)_{165}b$; MMP-9: Matrix Metalloproteinase-9; TNF-α: Tumor Necrosis Factor Alpha; GAD: Gestational Age in Days

Introduction

Preeclampsia is one of the oldest diseases on record [1], yet the etiology of preeclampsia is still unknown. Hence the quest for biomarkers continues with the hope that different biomarkers may help in understanding the underlying pathological process. Currently there is no suitable animal model to study the disease. Investigators have therefore relied on cell culture studies or have used fresh and cryogenically stored placental villous explants to identify biomarkers that might predict the syndrome [2,3].

A number of diverse theories on the causation of the disease have been put forward by many investigators over the years pertaining to oxidative stress, immunologic intolerance between the fetoplacental unit and the maternal tissue, and an angiogenic imbalance [4-7]. Regardless of the mechanisms, a two stage model of preeclampsia has been developed: in the first stage, reduced placental perfusion is thought to follow an abnormal implantation; and later failure in vascular remodeling is assumed to induce syncytiotrophoblast stress that interacts with maternal constitutional factors that trigger the maternal syndrome of preeclampsia [4-7].

Angiogenesis is one of the leading mechanisms in pregnancy that contributes to the exponential fetal growth throughout gestation; and placenta is the central offender in the pathophysiology of preeclampsia because delivery of the placenta can only reverse the syndrome. We, for several years, have investigated several placental angiogenic proteins independently in normal pregnancies and preeclampsia [8,9] in order to delineate the differences in the placental protein profiles between the two groups. In the present study we have simultaneously investigated four placental proteins: $VEGF_{165,}$ $VEGF_{165}b$, MMP-9 and TNF-α protein in normal pregnancies and preeclampsia in order to understand if simultaneous quantitation of the proteins can shed additional light in the pathophysiology of the vascular defect responsible for the disease. The hospital where the study was conducted primarily serves women of Hispanic and Black American population. Hence, in the present study, we have also examined the differences in the placental proteins between these two ethnic groups.

Methods

Women were not enrolled for the study. Placental tissues after clinical care following term deliveries from normotensive pregnancies that would otherwise have been discarded were collected under a protocol approved by the Institutional Human Subject Committee of the BronxCare Health System, Bronx, New York. The protocol also allowed collection of placentas from women with preeclampsia. The diagnosis of preeclampsia was made by the criteria of ACOG [10]. These women had no history of hypertension before pregnancy, their systolic and diastolic blood pressure was ≥ 140/90 mm Hg on at least two occasions 6 hours apart, and urinary protein ≥ 300 mg/24 hr. The IRB approved protocol allowed certain clinical information to be collected at the time of tissue collection without the identification of the patients' names and their medical record numbers. These included: maternal age, race, gestational age as determined by ultrasound and/or by the initial date of the last menstrual period, and method of delivery. The excluding criteria for both the study groups included pregnancies complicated with any symptom of infection, diabetes, hypertension, chronic renal disease, chronic peripheral vascular disease, multifetal gestation or with major fetal anomalies. To keep the preeclampsia and the normotensive group comparable the gestational age of both groups was limited from 37 to 42 weeks. Placental tissues were collected within 30 minutes of vaginal or cesarean deliveries. Placentas delivered overnight or when placenta sample collection could not be done within the 30 minute window, the placentas were not collected to keep the sample collection protocol consistent.

The placental membranes were removed. Placental tissues were then thoroughly washed and dissected in saline to collect free floating chorionic villi, not anchored to the basal plate nor emerging from the chorionic plate surface vessels. Sections of chorionic villi samples

from the same placenta were placed in individual cryovials bearing identical study number and were transported to the laboratory on ice. The chorionic villi samples from the same placenta were stored in individual freezer boxes at -80^{0}C until assay.

Commercially available Enzyme Immunoassay (EIA) kits were purchased from R&D Systems, Minneapolis, MN to determine the chorionic villi protein expressions of $VEGF_{165}$, $VEGF_{165}$b, MMP-9 and TNF-α. The four proteins were not analyzed from the same homogenate. $VEGF_{165}$ protein assays of all chorionic villi samples were carried out first. On the day of the assay, chorionic villi samples were taken out of the -80^{0}C freezer in chronological order the placental tissues were collected. The tissues were homogenized, then centrifuged at 13,000 rpm for 2 minutes and the supernatants were used for direct-sandwich EIA. When $VEGF_{165}$ protein assays were completed, $VEGF_{165}$b protein expression was carried out following the same chronological order as stated above, followed by MMP-9 protein and then TNF-α assays. Sensitivity of the assay kit used was 31.3 pg/ml for $VEGF_{165}$, 62.5 pg/ml for $VEGF_{165}$b, 31.3 pg/ml for MMP-9, and 15.6 pg/ml for TNFα, protein, respectively. Samples were analyzed in duplicates and the intra assay and inter assay variations for all proteins were < 10%.

Statistical analyses

Statistical evaluation of the data was carried out using SPSS^R statistical package version 26 (IBM Corporation, Armonk, NY). Normal distribution of the data was first tested and was found to be skewed. Chorionic villi tissue samples were grouped based on the presence or absence of preeclampsia and Independent T test was performed to assess difference in means. Spearman's Rank correlation coefficient test was applied to estimate the strength and direction of relationships between pairs of continuous variables. $P < 0.05$ was considered significant.

Results and Discussion

The rationale for selecting the four proteins was because the multistep process of placental angiogenesis begins with a rise in local angiogenic growth factors. Cytotrophoblasts during human pregnancy secrete several VEGF proteins, of which $VEGF_{165}$ is considered as the key contributor of the angiogenic process [11]. $VEGF_{165}$b, the alternate spliced form of $VEGF_{165}$, is generated from the same transcript and is present in appreciable amounts in human placenta during pregnancy [9,12]. After the rise in local angiogenic growth factors, breakdown of decidual basement membrane follows, that facilitates cytotrophoblasts migration and proliferation [13] along with simultaneous remodeling of the spiral arteries. Remodeling of the extracellular matrix additionally facilitates maternal and placental tissue morphogenesis and fetal development [14] and healthy pregnancies are dependent on matrix metalloproteinases (MMPs) for trophoblast cell invasion and embryo implantation [13,14]. Placental TNF-α is a cell signaling protein that during pregnancy induces the synthesis of matrix metalloproteinases (MMPs), allows the cytotrophoblasts to reach the spiral arteries deeper within the uterine decidua and augments the apoptosis of vascular smooth muscle cells surrounding the spiral arteries [15].

In this study, a total of 146 placentas were collected, 115 placentas from women who were normotensive throughout gestation and delivered at term; and 31 from women who developed preeclampsia during their pregnancy and delivered after 37 weeks of gestation. The demographic characteristics of women from whom placental samples were collected are shown in table 1. The results show that the mothers from whom the placentas were collected were of similar age groups. Compared to normotensive women, mothers with preeclampsia had significantly shorter gestation ($p = 0.001$). Both the systolic and diastolic blood pressures of women in the preeclampsia group were higher ($p = 0.001$). The mean newborn weights in the preeclampsia group was lower ($p = 0.001$). However, the placental weight was comparable between the two groups 459 ± 91g versus 417 ± 138g (Table 1). Race/ethnicity was self-reported and the distribution pattern showed that women in the study were 23.2% of Black American origin; 69.8% were of Hispanic American origin, and the remaining were of other ethnic groups. The two stage model of preeclampsia suggests that a poorly perfused placenta that occurs in Stage 1 is not sufficient to cause the maternal syndrome of preeclampsia [4-7]. Factors that are produced in poorly perfused placenta in

Stage 1 interact with maternal constitutional factors (genetic, behavioral or environmental) to result in Stage 2 [4-7]. Hence our study population of 69.8% Hispanic and 23.3% of Black Americans provided us with the opportunity to crudely explore if ethnic variations do contribute to the pathophysiology of preeclampsia. In the study, the number of placentas collected following spontaneous vaginal delivery and cesarean sections were 58 and 88, respectively.

Variables	Groups	N	Mean	Std. Dev	Sig. (2-tailed) Equal variance not assumed
GAD (in days)	Normotensive	115	276	7	0.02
	Preeclampsia	31	259	26	
Age (years)	Normotensive	115	28	6	0.194
	Preeclampsia	31	26	6	
Systolic BP (mm Hg)	Normotensive	115	120	15	0.000
	Preeclampsia	31	153	19	
Diastolic BP (mm Hg)	Normotensive	115	71	11	0.000
	Preeclampsia	31	92	13	
Placental weight (grams)	Normotensive	115	459	99	0.229
	Preeclampsia	31	426	141	
Newborn weight (grams)	Normotensive	115	3402	503	0.000

Table 1: *Characteristics related to mothers from whom placentas were collected.*

Results of independent T test that compared the protein expressions of the two groups are presented in table 2 and in figure 1. $VEGF_{165}$ and $VEGF_{165}b$ proteins were both identified in the third trimester of normotensive (n = 115) and preeclamptic (n = 31) placentas. The results showed that compared to the normotensive group the mean pro-angiogenic $VEGF_{165}$ protein expression in preeclamptic chorionic villi samples was significantly lower (p = 0.001) while the anti-angiogenic $VEGF_{165}b$ protein expression was significantly higher (p = 0.05), in contrast to the result of an earlier published report [12]. In this study a total of 115 normal and 31 preeclamptic placentas were analyzed to determine $VEGF_{165}$ and $VEGF_{165}b$ protein expressions; and the ELISA method used was specific to determine human $VEGF_{165}$ and $VEGF_{165}b$ protein independently. Moreover, chorionic villi samples were isolated from each placenta to determine particularly the $VEGF_{165}$ protein expressions of the cytotrophoblasts. MMP-9 and TNF-α protein expressions were both found to be significantly higher in the preeclampsia group (Table 2 and figure 1). Our findings are consistent with the report of Wang and Walsh [16] who reported a significantly higher expression of TNF-α protein in preeclamptic placentas. A higher concentration of TNF-α protein in the maternal circulation in preeclampsia was reported in another study. The investigators claimed that the higher concentration of TNF-α protein seen in preeclampsia was not caused by the disease but was the consequence of the disease because the higher expression of TNF-α only occurred after the clinical signs of disease were established [17]. Another study cited in the literature reported that TNF-α was not elevated in preeclamptic placenta despite its elevation in peripheral blood [18]. The expression of TNF-α protein in the study was determined using monoclonal antibody based ELISA kit just like ours but the earlier study differed from our study in several ways. First, the placental tissue used for ELISA included both maternal and fetal surfaces while in the present study placental chorionic villi were isolated to determine the TNF-α protein levels. Second, TNF-α protein concentrations in the previous study were expressed as TNF-α levels relative to total protein, while we had expressed our results as TNFα concentration per 100 mg wet tissue. The significantly higher concentrations of MMP-9 protein in preeclamptic placentas in this study (p = 0.036) is also in contrast with reports of lower concentration of MMP-9 protein in preeclamptic placentas [19]. Possible explanations for the discrepancies in the results could be due to the difference in the methods used, the type of tissue that were analyzed, serum versus placenta; or because the gestational age of the preeclampsia group did not match. Table 1 also shows that the maternal systolic and diastolic blood pressures in preeclampsia group were significantly

higher (p = 0.0001) but the newborn weights were significantly lower (p = 0.001); consistent with a previous report [19]. It needs to be underscored that the placental weights in the study were comparable between normal pregnancy and preeclampsia groups 459 ± 91g versus 417 ± 138g (Table 1), and the finding is in agreement with a large Norwegian study which reported placental weight in normal pregnancies (n = 304,875) to be 672g, and in term preeclampsia (n = 9,743) with gestational age greater 37 weeks to be 675g [20].

Variables	Groups	N	Mean	Std. Dev	Sig. (2-tailed) Equal variance not assumed
$VEGF_{165}$ (pg/100 mg tissue)	Normotensive	115	153.64	101.03	
	Preeclampsia	31	89.61	56.47	0.000
$VEGF_{165}b$ (pg/100 mg tissue)	Normotensive	115	341.67	231.11	
	Preeclampsia	31	448.03	267.79	0.05
MMP-9 (ng/100 mg tissue)	Normotensive	115	26.08	16.30	
	Preeclampsia	31	33.11	16.00	0.036
TNF-α (pg/100 mg tissue)	Normotensive	115	36.84	24.72	
	Preeclampsia	31	57.48	51.20	0.037
	Preeclampsia	31	2723	867	

Table 2: *Chorionic villi protein expressions.*

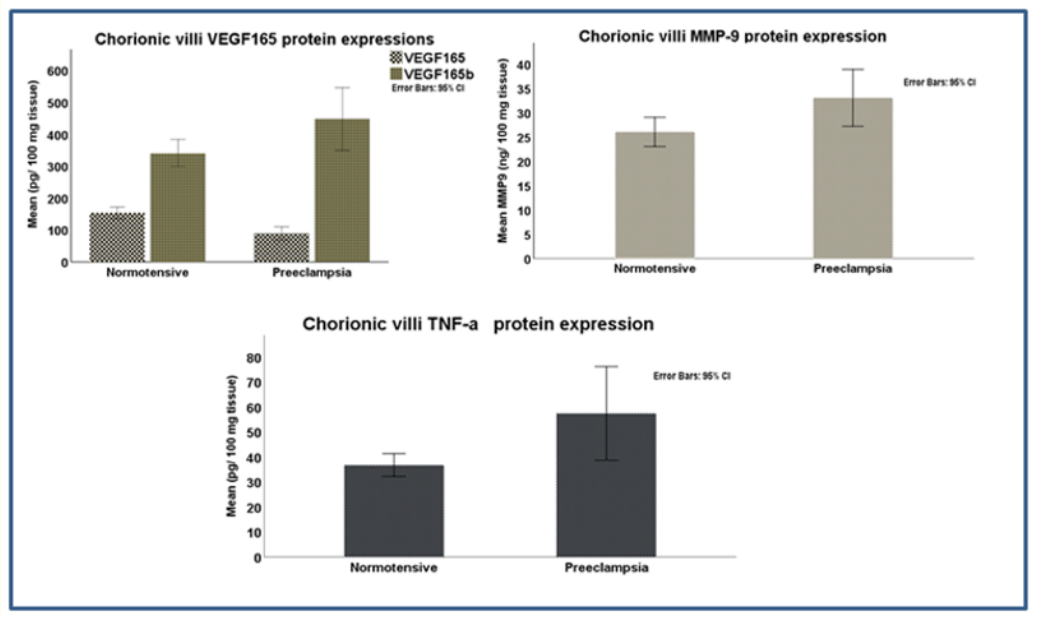

Figure 1: *Chorionic villi $VEGF_{165}$, $VEGF_{165}b$, MMP-9 and TNF-α protein expression of the two study groups are shown here. Expression of each chorionic villi protein significantly differed between normal pregnancy (n = 115) and preeclampsia (n = 31) groups.*

In this study, Spearman Rank correlation coefficient test was used to determine the correlations between the ten studied variables. The correlations in the normotensive and preeclampsia groups were separately analyzed. Table 3 depicts the correlations between the studied variables in normal placentas (n = 115). The positive correlation in the expressions of $VEGF_{165}$ and $VEGF_{165}b$ placental proteins (r = 0.280, p = 0.002), suggest a synergistic action between the two proteins in the third trimester of human pregnancy, consistent with a previously published results [12]. To our knowledge placental expression of $VEGF_{165}$ and $VEGF_{165}b$ proteins in human pregnancy has not been reported elsewhere other than the one already cited in the manuscript. Table 3 also shows that in normal pregnancy the systolic and diastolic blood pressures of the mothers from whom the placentas were taken were significantly correlated (r = 0.571, p = 0.000). A significant positive correlation was also noted between placental and newborn weights (r = 0.557, p = 0.000). A relevant feature of Table 3 is the negative correlation seen between $VEGF_{165}$ and MMP-9 protein (r = -0.185, p = 0.047) (Table 3). Regulation of angiogenesis is a multistep process that requires activation and proliferation of endothelial cells and degradation of extracellular matrix, enabling the proliferating cells to invade the surrounding tissue. During tumorigenesis, basement membrane destruction is an essential step for tumor invasion and metastases, and cellular invasion is dependent on MMP-9 protein [21]. A transgenic mouse model of multistage carcinogenesis showed that MMP-9 protein can explicitly render quiescent normal pancreatic islets cells to become angiogenic, by inducing the release of VEGF protein [22]. The authors confirmed that these results could not be replicated when other molecules were used. The study additionally showed that when the activity of MMP-9 protein was inhibited by MMP-9 inhibitors, the angiogenic switching was reduced, concomitant with a decrease in the number of tumors and in tumor growth. Genetic ablation of MMP-9 produced similar results [22]. To summarize, the study showed that during tumorigenesis it is MMP-9 protein that induced the release of VEGF to trigger the angiogenic process. Cytotrophoblasts during pregnancy similarly mirrors to a certain extent the behavior of malignant cells. The difference between tumorigenesis and pregnancy is that while the invasion and angiogenic processes in tumorigenesis continues, in pregnancy these processes after a certain period of time need to cease. Hence, the significant negative correlation seen in the third trimester of normal pregnancy between $VEGF_{165}$ and MMP-9 protein (r = -0.185, p = 0.047, Table 3) may reflect an inherent physiological phenomenon in human pregnancy to terminate the angiogenic process.

Variables		$VEGF_{165}$	$VEGF_{165}b$	MMP-9	TNF-α	GAD	Sys Pr	Dia Pr	Pl Wt	NB Wt
$VEGF_{165}$ (pg/100 mg tissue)	Corr Coeff	1.000	0.261	-0.185	0.137	0.031	0.019	-0.042	0.009	-0.037
	Sig (2-Tailed)		0.005	0.047	0.143	0.744	0844	0.653	0.925	0.697
	N	115	115	115	115	115	115	115	115	115
$VEGF_{165}b$ (pg/100 mg tissue)	Corr Coeff	0.261	1.000	-0.020	-0.002	-0.028	0.020	0.083	0.049	0.128
	Sig (2-Tailed)	0.005		0.834	0.979	0.765	0.832	0.376	0.603	0.173
	N	115	115	115	115	115	115	115	115	115
MMP-9 (ng/100 mg tissue)	Corr Coeff	-0.185	-0.020	1.000	-0.080	-0.031	-0.064	-0.114	-0.060	-0.146
	Sig (2-Tailed)	0.047	0.834		0.397	0.743	0.494	0.226	0.528	0.119
	N	115	115	115	115	115	115	115	115	115
TNF-α (pg/100 mg tissue)	Corr Coeff	0.137	-0.002	-0.080	1.000	-0.030	0.163	0.118	0.053	0.078
	Sig (2-Tailed)	0.143	0.979	0.397		0.754	0.081	0.209	0.574	0.406
	N	115	115	115	115	115	115	115	115	115
Gestationa age in days	Corr Coeff	0.031	-.028	-0.031	-0.030	1.000	-.007	0.096	-0.023	.187
	Sig (2-Tailed)	0.744	0.765	0.743	0.754		0.938	0.308	0.810	0.045
	N	115	115	115	115	115	115	115	115	115
Systolic Blood Pressure (mm Hg)	Corr Coeff	0.019	0.020	-0.064	0.163	-0.007	1.000	.571	0.008	0.116
	Sig (2-Tailed)	0.844	0.832	0.494	0.081	0.938		0.000	0.930	0.217
	N	115	115	115	115	115	115	115	115	115
Diastolic Blood Pressure (mm Hg)	Corr Coeff	-0.042	0.083	-0.144	0.118	0.096	.571	1.000	-0.110	-0.051
	Sig (2-Tailed)	0.653	0.376	0.226	0.209	0.308	0.000		0.241	0.585
	N	115	115	115	115	115	115	115	115	115
P WT Weight (gms)	Corr Coeff	0.009	0.049	-0.060	0.053	-0.023	0.008	-0.110	1.000	0.557
	Sig (2-Tailed)	0.925	0.603	0.528	0.574	0.810	0.930	0.241		0.000
	N	115	115	115	115	115	115	115	115	115
NB WT Weight (gms)	Corr Coeff	-0.037	0.128	-0.146	0.078	0.187	0.116	-0.051	0.557	1.000
	Sig (2-Tailed)	0.697	0.173	0.119	0.406	0.045	0.217	0.585	0.000	
	N	115	115	115	115	115	115	115	115	115

Table 3: *Spearman's rank correlation coefficient results of studied variables in normal pregnancy. Corr Coeff: Correlation Coefficient; GAD: Gestational Age in Days; Sys Pr: Systolic Blood Pressure; Dia Pr: Diastolic Blood Pressure; Pl WT: Placental Weight; NB WT: Newborn Weight.*

The Spearman's Rank coefficient correlation data between the 10 studied variables in preeclamptic placentas are presented in table 4. In contrast to normotensive pregnancies, the two $VEGF_{165}$ and $VEGF_{165}b$ proteins in preeclamptic placentas were not correlated. This lack of correlation between $VEGF_{165}$ isoforms we interpret as an imbalance in $VEGF_{165}$ isoforms in preeclampsia that disrupts the normal angiogenic course. Since the placental weight between normal pregnancy and preeclampsia was comparable (Table 1) hence, it suggests that the angiogenic process in preeclamptic placenta continued, but the intracellular signaling mechanisms driving angiogenesis must have been different. The synergistic action between the two $VEGF_{165}$ proteins in normal pregnancy may act as a directional signal influencing capillary growth. The synergistic actions of the two $VEGF_{165}$ isoforms, we suggest, may modify the placental angiogenesis in favor of the sprouting type that allowed new branches to be formed on existing blood vessels and maximized the amount of blood that flowed through, to result in healthy newborns with mean weight of 3402g. The imbalance in the two $VEGF_{165}$ proteins in preeclampsia, we hypothesize, may be an angiogenic biomarker that triggered angiogenesis of the intussusceptive type. In contrast to sprouting angiogenesis, during intussusceptive angiogenesis each blood vessel splits into two [23]; the caliber of each blood vessels become reduced to half its size, and progressively decrease further with each subsequent division [23]. Such progressive decrease in blood vessel diameter, we hypothesize ultimately leads to ischemia and vascular dysfunction consistent with preeclampsia. In the present study, the MMP-9 protein expression is significantly higher in preeclamptic placenta (Table 2). If the concept of intussusceptive angiogenesis holds, the angiogenic process will yield capillaries that are much reduced in diameter. Hence, to intensify the amount of blood that flows to the fetus, because the fetal demand surges in the third trimester; the capillary plexus need to grow at a much faster rate in comparison to sprouting angiogenesis. The significantly higher MMP-9 protein expression seen in preeclamptic placentas (Table 1), we suggest, supports the extracellular matrix to rapidly degrade to make room for the capillary plexus to grow. A significant correlation between placental weight and placental TNF-α protein was seen in preeclampsia ($r = 0.385$, $p = .032$), underscoring the support of locally produced cytokine in placental health in preeclamptic condition. Additionally, in preeclampsia, GAD was significantly correlated to placental and newborn weights ($r = 0.508$, $p = 0.004$; $r = 0.781$, $p = 0.001$, respectively). The finding led us to suggest that perhaps the newborn outcome could be better if the gestational age of the women in the preeclampsia group is allowed to stretch.

Variables		$VEGF_{165}$	$VEGF_{165}b$	MMP-9	TNF-α	GAD	Sys Pr	Dia Pr	Pl Wt	NB Wt
$VEGF_{165}$ (pg/100 mg tissue)	Corr Coeff	1.000	0.291	0.097	0.304	-0.255	-0.314	-0.022	0.148	-0.138
	Sig (2-Tailed)		0.113	0.605	0.096	0.165	0.085	0.907	0.428	0.459
	N	31	31	31	31	31	31	31	31	31
$VEGF_{165}b$ (pg/100 mg tissue)	Corr Coeff	0.291	1.000	0.027	0.036	-0.299	0.102	0.181	-0.059	-0.419
	Sig (2-Tailed)	0.113		0.887	0.848	0.103	0.584	0.329	0.754	0.019
	N	31	31	31	31	31	31	31	31	31
MMP-9 (ng/100 mg tissue)	Corr Coeff	0.097	0.027	1.000	0.049	0.071	0.175	0.269	0.303	0.022
	Sig (2-Tailed)	0.605	0.887		0.793	0.706	0.346	0.143	0.097	0.908
	N	31	31	31	31	31	31	31	31	31
TNF-α (pg/100 mg tissue)	Corr Coeff	0.304	0.036	0.049	1.000	-0.077	-0.313	0.066	0.385	0.033
	Sig (2-Tailed)	0.096	0.848	0.793		0.680	0.087	0.724	0.032	0.859
	N	31	31	31	31	31	31	31	31	31
Gestational age in days	Corr Coeff	-0.255	-0.299	0.071	-0.077	1.000	0.009	-0.252	0.508	0.781
	Sig (2-Tailed)	0.165	0.103	0.706	0.680		0.961	0.172	0.004	0.000
	N	31	31	31	31	31	31	31	31	31
Systolic Blood Pressure (mm Hg)	Corr Coeff	-0.314	0.102	0.175	-0.313	0.009	1.000	.744	0.031	-0.076
	Sig (2-Tailed)	0.085	0.584	0.346	0.087	0.961		0.000	0.867	0.683
	N	31	31	31	31	31	31	31	31	31
Diastolic Blood Pressure (mm Hg)	Corr Coeff	-0.022	0.181	0.269	0.066	-0.252	0.744	1.000	-0.087	-0.257
	Sig (2-Tailed)	0.907	0.329	0.143	0.724	0.172	0.000		0.643	0.163
	N	31	31	31	31	31	31	31	31	31
P WT Weight (gms)	Corr Coeff	0.148	-0.059	0.303	0.385	0.508	0.031	0.087	1.000	0.592
	Sig (2-Tailed)	0.428	0.754	0.097	0.032	0.004	0.867	0.643		0.000
	N	31	31	31	31	31	31	31	31	31
NB WT Weight (gms)	Corr Coeff	-0.138	-0.419	0.022	0.033	0.781	-0.076	-0.257	0.592	1.000
	Sig (2-Tailed)	0.459	0.019	0.908	0.859	0.000	0.683	0.163	0.000	
	N	31	31	31	31	31	31	31	31	31

Table 4: *Spearman's rank correlation coefficient results of studied variables in preeclampsia.*
Corr Coeff: Correlation Coefficient; GAD: Gestational Age in Days; Sys Pr: Systolic Blood Pressure; Dia Pr: Diastolic Blood Pressure; Pl WT: Placental Weight; NB WT: Newborn Weight.

In this study, of the total 146 placentas that were collected, 101 placentas were of mothers of Hispanic American origin (69.8%), 34 were from mothers of Black American descent, and the remaining 11 were from other ethnic backgrounds. Out of 34 women from the Black American ethnic group, 15 had preeclampsia, and out of 101 women from the Hispanic American ethnic group 14 had preeclampsia. However, we need to interpret the results of this study as Black American women being at a higher risk of preeclampsia compared to Hispanic mothers with caution. The reason being, that in the study there was a limitation in our tissue collection process. We had a 30 minute window limit from the time of delivery and placental sample collection. Placentas that were delivered outside this 30 minute window or were delivered overnight were not collected. And perhaps this limitation might have affected the result. Nonetheless, higher incidence of preeclampsia among Black American women is consistent in other reports [24,25]. In a recent study that included 6000 women of racially diverse groups it was stated that race alone was unlikely to account for the higher rates of preeclampsia among Black women, and other biological, social and cultural factors may contribute to the process [26].

Previous studies examining the risk factors of preeclampsia have compared ethnic differences in maternal blood constituents [27,28]. To our knowledge this is perhaps the first study that investigated the differences in placental angiogenic proteins between two ethnic groups, Black and Hispanic population. Figure 2-4 summarize the differences in the studied variables between the two ethnic groups and reveal that ethnic differences in placental angiogenic proteins do exist. Black women who had preeclampsia (n = 15) showed a significant positive correlation between placental $VEGF_{165}$ and TNF-α protein (r = 0.571, p = 0.026). In an *in vitro* study with HUVEC cells, varying the concentration of VEGF available to the growing vessel was shown to provide the control to regulate the diameter of the vessels. The authors suggested that VEGF may dilate vessels as a result of thinning of the endothelial cells or by recruiting endothelial cells within them [29]. During pregnancy, nitric oxide synthase activity is highest in the villous trophoblast of term placenta [30]. In an *in vivo* model, it was shown that application of 100 ng/ml of TNF-α protein added to cerebrospinal fluid to rats caused marked progressive nitric oxide-mediated dilation of cerebral arterioles with a maximum increase in diameter at 4 hours [31]. In preeclampsia, the caliber of the blood vessels is significantly reduced; and the trophoblast invasion of the spiral arteries is incomplete, hence, the endothelial and smooth muscle cells are retained within the spiral arteries rather than being replaced by cytotrophoblasts. Based on the findings of an upregulation of TNF-α protein in preeclampsia (Table 2), along with a significant positive correlation seen between $VEGF_{165}$ and TNF-α proteins (r = 0.571, p = 0.026) we suggest, that the upregulation in TNF-α protein could be a compensatory mechanism in preeclampsia to assist the reduced caliber blood vessels to dilate; so that greater amounts of blood can be transported to the fetus, to keep up with the increase in fetal demand of the third trimester. Black women who had preeclampsia also had significant correlations between MMP-9 protein and GAD (r = 0.592, p = 0.02); MMP-9 protein and placental weight (r = 0.775, p = 0.001) and MMP-9 protein and newborn weight (r = 0.568, p = 0.027). Among Black American with preeclampsia, the chorionic villi anti-angiogenic $VEGF_{165}b$ protein expression was significantly higher (p = 0.049) compared to their normotensive group. Women of Hispanic origin who had preeclampsia were significantly younger (28 ± 6 years versus 25 ± 4 years, p = 0.03) and the chorionic villi pro-angiogenic $VEGF_{165}$ protein expression was significantly lower (p = 0.01) compared to the normotensive group. Thus, the findings of this study revealed that the correlations between the studied placental proteins differed between the two ethnic groups. Overall, the data of the present study supports the notion that ethnic variations can impact the maternal constitutional factors differently in triggering the maternal syndrome of preeclampsia. We think a greater understanding of the relationship of known biomarkers and ethnicity of women with preeclampsia may provide fresh insight into the pathophysiology of the disease.

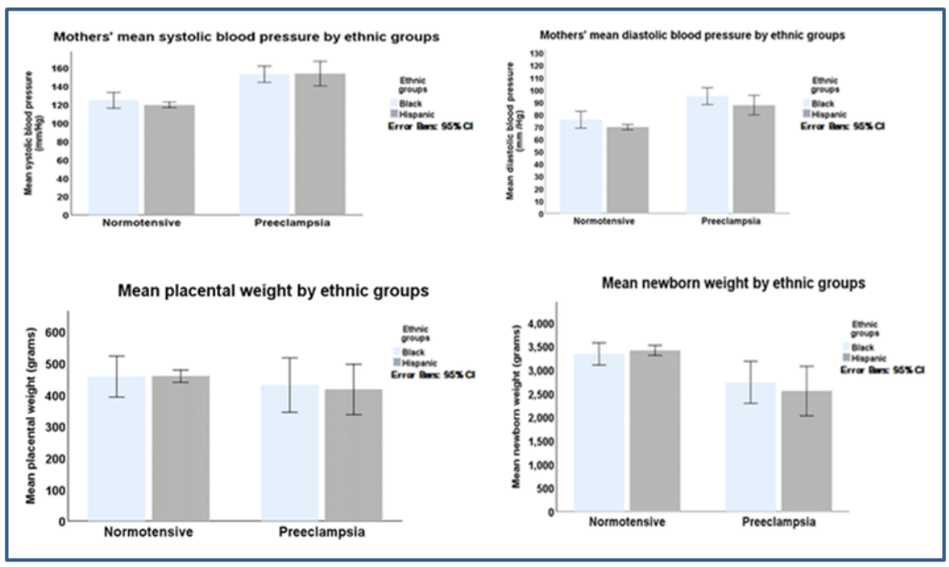

Figure 2: *Differences in the mother's and diastolic blood presure; and placental weight and newborn weights of the two ethnic groups are shown. The ethnic groups from whom the placentas were collected comprised of: 19 Black women in the normotensive group and 15 Black women who had preeclampsia; and 87 Hispanic women in the normotensive group and 14 Hispanic women who had preeclampsia.*

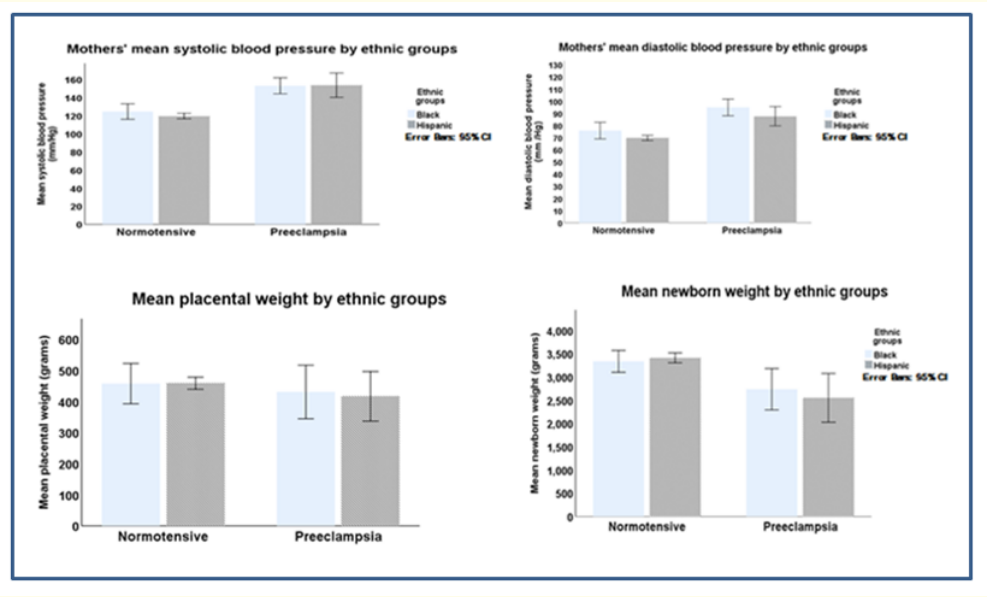

Figure 3: *Differences in the mothers' systolic and diastolic blood pressure; and placental weight and newborn weights of the two ethnic groups are shown. The ethnic groups from whom the placentas were collected comprised of: 19 Black women in the normotensive group and 15 Black women who had preeclampsia; and 87 Hispanic women in the normotensive group and 14 Hispanic women who had preeclampsia.*

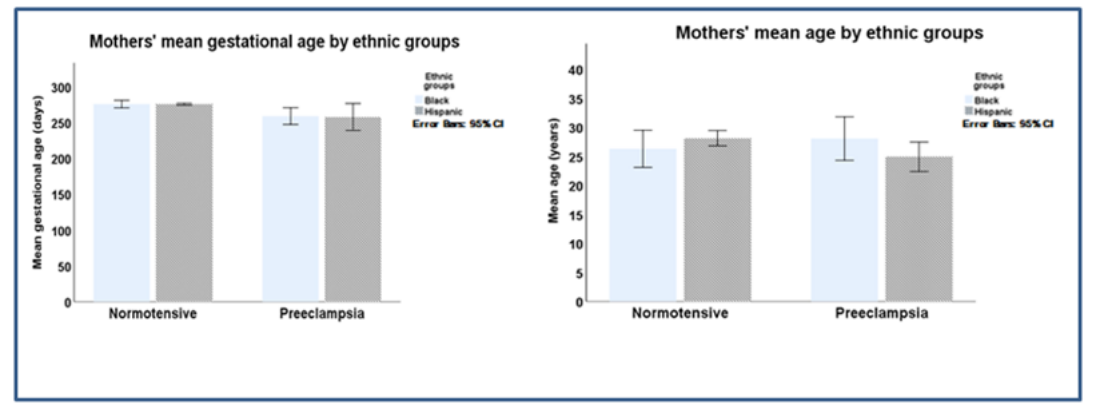

Figure 4: *Differences in Mothers' gestational age and mothers' age between the two ethnic groups are shown here. The ethnic groups from whom the placentas were collected comprised of: 19 Black women in the normotensive group and 15 Black women who had preeclampsia; and 87 Hispanic women in the normotensive group and 14 Hispanic women who had preeclampsia.*

In this study, the higher number of placentas collected following cesarean sections (n = 88) manifests the ease of collecting placental samples in a timely manner from a scheduled cesarean section than collecting placentas following spontaneous vaginal delivery (n = 58). Independent T test results revealed that women who had cesarean section were significantly older than those who delivered vaginally (29 ± 6 years versus 25 ± 6 years, p = 0.01). The systolic and diastolic blood pressures of the mothers who delivered vaginally were 131 ± 24 mm Hg and 79 ± 15 mm Hg, respectively; whereas the systolic and diastolic blood pressures of mothers who delivered by cesarean sections were 124 ± 18 mm Hg and 73 ± 13 mm Hg, respectively, the differences were statistically significant p = 0.041 and p = 0.012, respectively). Expressions of the four placental proteins were not affected by the methods of delivery used. In the study, 72 placentas were from mothers who had delivered female newborns and 74 were from mothers who had delivered male newborns. The studied variables were examined to determine if the gender of the fetus had any effect on the studied variables. Results showed that in mothers who carried a male fetus, the placental weight was 434 ± 105g and in mothers who carried a female fetus the placental weight was 470 ± 112g and the differences in placental weight was statistically significant (p = 0.05). Differences in the other studied variables were not significant.

Conclusion

In the present study we investigated whether comparison of chorionic villi angiogenic protein expression patterns between normotensive pregnancies and preeclampsia could elucidate the vascular abnormality seen in preeclampsia. Significant differences were found in: a) mean $VEGF_{165}$/ $VEGF_{165}$b ratios (p = 0.0001), b) mothers' systolic and diastolic blood pressure (p = 0.000, p = 0.000, respectively); and in newborn weights (p = 0.00) between normal pregnancies and preeclampsia. Yet, the placental weight between the two groups was comparable suggesting that the intracellular signaling mechanisms driving angiogenesis in the two studied groups may have been different. The synergistic action between the two $VEGF_{165}$ proteins in normal pregnancy may act as a directional signal influencing capillary growth modifying the placental angiogenesis in favor of the sprouting type. Formation of new branches on the existing blood vessels may have maximized the amount of blood flow, resulting in healthy newborns with mean weight of 3402g. The imbalance in the two $VEGF_{165}$ proteins in preeclampsia may be an angiogenic biomarker that triggered angiogenesis of the intussusceptive type. Since during intussusceptive angiogenesis each blood vessel splits into two, the caliber of each blood vessel progressively decrease. Such progressive decrease in blood vessel diameter, we hypothesize ultimately leads to ischemia and vascular dysfunction consistent with preeclampsia. If the concept of intussusceptive angiogenesis holds, the angiogenic process will yield capillaries that are much reduced in diameter. Hence, to intensify the amount of blood that flows to the fetus, as the fetal demand surges in the third trimester; the capillary plexus need to grow at a much faster rate in comparison to sprouting angiogenesis. It may be suggested that the higher expression of chorionic villi MMP-9 protein seen in the present study in preeclampsia is ~~as~~ a compensatory mechanism to support intussusceptive angiogenesis and rapid degradation the extracellular matrix to allow the capillary plexus to grow. Higher expression of TNF-α protein in preeclamptic placenta, we suggest, could also be a compensatory mechanism, to assist the spiral arteries to dilate; so that greater amounts of blood can be transported to the fetus, to keep up with the surge in fetal demand of the third trimester.

Acknowledgement

The study was funded by the Residency Program Grant for the BronxCare Health System. The authors thank the Residents and staff members of the Department of Obstetrics and Gynecology at BronxCare Health System for their support in carrying out this study.

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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Volume 11 Issue 9 September 2022