

Pre-eclampsia is more than a vascular disease

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Pre- eclampsia is more than a vascular disease

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PRE- ECLAMPSIA IS MORE THAN A VASCULAR DISEASE

DISSERTATION

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ABBREVIATIONS

ADMA	asymmetric dimethylarginine
CoQ ₁₀	Co-enzyme Q ₁₀
G-CSF	Granulocyte colony stimulating factor
HCy	Homocysteine
IF	Interferon
IL	Interleukin
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory Protein
MTHFR	Methylenetetrahydrofolate reductase
NO	Nitric oxide
NOS	Nitric oxide synthase
PE	Pre- eclampsia
PLGF	Placental growth factor
sFlt-1	Soluble receptor fms-like tyrosin kinase 1
SGA	Small for gestational age
SNPs	Single nucleotide polymorphisms
TNF-R	Tumor necrosis factor receptor
TNF- α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

CHAPTER 1

Introduction

Pre-eclampsia (PE) is a serious issue in female reproductive medicine [1]. To date, despite attempts at intervention it is still a leading cause of maternal and fetal morbidity and mortality [1]. Indeed, to date, there is no treatment for this disease other than the termination of pregnancy, resulting in an increased rate of iatrogenic preterm births. Hence research has focused on prevention and the search for predictive markers that will provide early treatment or intervention.

The Etiology of PE is still unknown; however clinical and *in vitro* data obtained at various stages of pregnancy have provided insights to the various biochemical abnormalities found in this disease [2]. In general, abnormalities originate in the placenta and have been related to vessels (maternal and placental), anti-oxidation, inflammation and genetics. The role of each will be reviewed and discussed in this thesis in order to further elucidate biochemical and molecular mechanisms involved in the pathogenesis of this disease.

A. EPIDEMIOLOGY OF PRE- ECLAMPSIA AND PATHOGENESIS

PE is a frequent complication of pregnancy that accounts for 5 to 14% of all deliveries, and causes adverse maternal and perinatal outcomes [3,4]. Although the exact cause of PE remains undetermined, key maternal and placental pathogenic factors include:

- Immunologic factors,
- Abnormal placental implantation,
- Genetic and environmental factors, and
- Cardiovascular and exaggerated inflammatory changes [3,5-7].

B. IS PRE- ECLAMPSIA PREDICTION FEASIBLE?: THE ROLE OF BIOCHEMICAL MARKERS

Reduced placental perfusion at early stages of pregnancy is a key event in the development of PE [8]. Defective trophoblastic invasion of the uterine spiral-arteries and arterioles leads to incomplete vascular remodeling and impaired utero-placental blood flow. Consequently, the placenta becomes ischemic and hypoxic and secretes into the maternal plasma an array of factors that are implicated in inducing generalized endothelial cell activation and vascular dysfunction that elicit the maternal clinical syndrome [10,11].

Although the exact mechanisms involved remain unclear, vascular dysfunction found in PE is likely to be a consequence of reduced maternal circulating angiogenic factors [12,13] and increased levels of placental debris [14], reactive oxygen species [15], pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF- α), interleukin 6 (IL-6) and interleukin 8 (IL-8)] [16,17] and anti-angiogenic factors [18,19].

B.1. Reduced angiogenic factors

Women with PE display abnormal decidual and placental villous vasculature. In one study [12], serum maternal concentrations of vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) were measured in normal pregnancies and those complicated by isolated idiopathic small-for-gestational-age (SGA) newborn infants, PE alone, or PE with SGA newborn infants at the time of clinical disease and before the onset of clinical signs. Serum VEGF and PLGF levels were found reduced in abnormal pregnancies as compared to control subjects. These findings were observed as early as 15 to 19 weeks of gestation in PE with SGA newborn infants. Authors concluded that decreased PLGF production results in abnormalities of placental angiogenesis through direct and indirect effects on other vascular growth factors [12].

B.2. Increased placental debris

It has been proposed that during PE there is a systemic inflammatory response involving both leucocytes and endothelium. This response is also present during normal pregnancy, but in a milder form. The inflammatory stimulus is most likely to come from the placenta. In normal pregnancies syncytiotrophoblast apoptotic debris is shed into the maternal circulation and contribute to the suppression of Th1 responses; however during PE this response is increased [14].

B.3. Increase of oxygen species/decreased anti-oxidation

During PE there is an observed increase in the rate of lipid peroxidation, increased lipid availability, and decrease of several antioxidants such as alpha tocopherol, ascorbate, beta carotene and selenium [20]. In accordance to this, a significant decrease in plasma coenzyme Q₁₀ (CoQ₁₀) in women with PE has recently been reported [21]. CoQ₁₀ is a part of the non-enzymatic defense system against oxygen species (antioxidative function) with mitochondrial complexes I and III reaction mechanisms playing a key role in electron transport [22]. One study performed in Ecuador found lower plasma and higher placental CoQ₁₀ content among PE women living in Quito, Ecuador (2,800 meters above sea level) when compared to normal pregnancies [23].

B.4. Increased pro-inflammatory cytokines

Generalized maternal endothelial cell dysfunction appears to be the final underlying problem which clinical signs and symptoms. This scenario is presumed to be caused, directly or indirectly, by one or more circulating factors derived from the placenta. Reports indicate that TNF- α may play an important role in causing endothelial activation [16]. One report measured plasma levels of TNF- α , IL-6 and both forms of soluble TNF

receptors (p55 and p75 TNF-R) in 31 women complicated with PE and 31 controls matched for age, parity and gestational age. All measured analytes were found to be significantly higher in PE women than in controls with a wide variation in levels between PE individuals. There was a correlation between IL-6 and TNF- α or TNF-R levels and between TNF- α and TNF-R levels. Nevertheless, when PE women were subdivided on the basis of the severity of their disease, median plasma concentration values of IL-6, TNF- α and TNF-R were all higher in the group with lower platelet counts [16]. Results of this study are consistent with the concept that the maternal syndrome of PE is associated with excessive release of TNF- α into the circulation which causes endothelial dysfunction.

During PE the immune system is changed with an increased innate activity and there is a hypothesis of a shift towards Th1-type immunity. Jonsson et al [17] measured a spectrum of soluble immunological factors that denote the different aspects of immune activation in the sera of third trimester women with PE and compared with levels in normal pregnant women. The array of pro- and anti-inflammatory cytokines included: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p40, IL-13, IL-15, IL-17, IFN- α , IFN- γ , TNF- α , G-CSF, MIP-1 α , MIP-1 β , MCP-1, eotaxin and RANTES which were measured with multiplex technology. Soluble CD14 and soluble IL-4 receptor levels were measured with ELISA. PE women displayed significantly higher levels of circulating IL-6 ($p=0.002$), IL-8 ($p=0.003$) and soluble IL-4R ($p=0.037$), compared to women with normal pregnancies [17]. Hence, the data supports the hypothesis of increased inflammatory responses in PE, demonstrated by the increased levels of IL-6 and IL-8. Higher levels of soluble IL-4 receptor may partly support the hypothesis of a Th1 shift during PE [17].

B.5. Increased anti-angiogenic factors

Soluble receptor fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) are potent anti-angiogenic factors that are elevated in the plasma of women experiencing PE [24,25]. Increased levels of these factors are thought to contribute to maternal vascular dysfunction by binding to and thus reducing circulating levels of free VEGF and PLGR [26].

B.6. Endothelial dysfunction

Placental ischaemia occurring during PE leads to the release of soluble placental factors, many of which, as already discussed, are classified as anti-angiogenic or pro-inflammatory. Once these factors reach the maternal circulation, they cause widespread activation and dysfunction of the maternal vascular endothelium that results in enhanced formation of endothelin-1 and superoxide, increased vascular sensitivity to angiotensin II and decreased formation of vasodilators such as nitric oxide (NO) [27].

NO is an intracellular gaseous messenger synthesized by NO synthase (NOS) from L-arginine and oxygen. NO exerts diverse biological functions in several physiological and pathological processes, especially in vascular pathophysiology [28]. In relation to pregnancy, two important roles have been identified: a) NO produced by syncytiotrophoblast-derived endothelial cells causes dilation of the human placental vasculature [29], and may act as a paracrine agent for the maintenance of uterine quiescence during pregnancy; and b) local placental NO generation seems to be essential to promote cytotrophoblast endovascular invasion, an essential feature of normal placentation [30].

Studies addressing NO levels in women with PE are contradictory some indicating higher [31], others lower levels [32]. In one previous Ecuadorian report, women with PE exhibited higher NO maternal plasma levels [33]. Contradictory results could probably be due to differences in the measurement methods and/or the metabolite analyzed. Nevertheless, there is agreement in that PE is associated with altered NO production and/or activity.

C. THE ROLE OF GENETICS IN THE PATHOGENESIS OF PE

Although it is accepted that PE has a placental origin and is multifactorial, a role for underlying genetic predisposition is gaining much interest. Indeed, genetic factors influence all of the pathophysiological mechanisms to date proposed [34]. The inherited nature of PE has been known for many years, and extensive genetic studies have been undertaken in this area [34]. It is now accepted that PE is a complex genetic disorder, occurring as the result of variants at different loci, which individually have small effects but collectively contribute to an individual's susceptibility to disease. It is probable that no single gene or variant will be identified that is responsible for all cases of PE, although different variants may prove to be associated with subsets of disease, such as early onset PE with fetal growth restriction.

To date, more than 70 candidate genes have been selected on the basis of prior biological knowledge of the pathological changes occurring in PE. These can be grouped based on their suggested pathophysiological mechanisms: vasoactive proteins, thrombophilia and hypofibrinolysis, oxidative stress and lipid metabolism, endothelial injury, and immunogenetics (Table 1) [35]. Despite vast research, to date no candidate gene has been universally accepted as a unique causal gene for PE. This may in part be due to ethnic variations within study populations and inconsistency in the definition of PE, and perhaps as a major reason the fact that the majority of candidate gene studies have been grossly underpowered to detect variants with small effects [34].

Table 1. Candidate genes and predominant polymorphisms implicated in PE pathogenesis*

Proposed underlying mechanism	Name of the gene name	Gene symbol	Polymorphism
Vasoactive proteins	Angiotensinogen	<i>AGT</i>	235 Met > Thr
	Angiotensin converting enzyme	<i>ACE</i>	I/D intron 16
Thrombophilia and hypofibrinolysis	Factor V Leiden	<i>F5</i>	506 Gln > Arg
	Methylenetetrahydrofolate reductase	<i>MTHFR</i>	C667T
	Prothrombin	<i>F2</i>	G20210A
	Plasminogen activator factor-1	<i>SERPINE 1</i>	Promoter insertion/deletion
	Integrin glycoprotein IIIa	<i>GPIIIA</i>	C98T
Oxidative stress and lipid metabolism	Apolipoprotein E	<i>APOE</i>	C886T
	Microsomal epoxide hydrolase	<i>EPHX</i>	113Tyr > His
	Glutathione-S-transferase	<i>GST</i>	A313G
Endothelial function	Endothelial nitric oxide synthase 3	<i>eNOS3</i>	298 Glu > Asp
	Vascular endothelial growth factor receptor I	<i>VEGFR 1</i>	TG repeat
	Vascular endothelial growth factor	<i>VEGF</i>	C936T
Immunogenetics	Tumour necrosis factor α	<i>TNF</i>	G-308 ^a
	Interleukin 10	<i>IL10</i>	G1082A

*Adapted from Mutze et al [35].

Single-nucleotide polymorphisms (SNPs) are variations of the genome sequence, which may modify biological responses, and the risk of certain diseases. Numerous SNPs have been studied in PE patients [36,37]. An interesting studied gene in relation to PE is the methylenetetrahydrofolate reductase (MTHFR) gen. The enzyme coded this gene is critical for homocysteine (HCy) metabolism, catalyzing the NADPH-linked reduction of 5,10-MTHF to 5-MTHF, and subsequently the vitamin B12-dependent methylation of HCy to methionine [38]. A reduction in MTHFR levels or activity by specific gene mutations induces mild to moderate increases in plasma HCy levels [39]. Although several mutations of the MTHFR gene have been described in relation to PE, the most frequent ones include: the alanine-to-valine C677T [40], and the glutamate-to-alanine A1298C [41].

Genetic research offers an attractive strategy for studying the pathogenesis of PE as it avoids the ethical and practical difficulties of conducting basic science research during the preclinical phase of PE when the underlying pathological changes occur.

AIMS & OUTLINE OF THE THESIS

The aim of this thesis was to assess various biochemical and molecular markers related to vascular, anti-oxidative, endothelial, inflammatory and genetic abnormalities found in gestations complicated with PE. **Chapter 2**, presents one study that evaluates vascular

homeostasis and inflammation during PE. Plasma levels of two soluble anti-angiogenic factors (sFlt-1 and sEng) and four pro-inflammatory cytokines (IL-6, IL-8, G-CSF and TNF- α) were measured in nulliparous women complicated with PE and levels compared to those found in normal nulliparous gestations. The results suggest that increased sFlt-1 and Eng levels in maternal plasma are consistent with vascular dysfunction found in gestations complicated with pre- eclampsia. Contrary to expected PE women displayed lowered IL-8 and G-CSF levels. This new finding is analyzed in the discussion.

In a previous research we found lower maternal plasma CoQ₁₀ levels in women complicated with PE [21]. As a continuation, **Chapter 3**, presents a study that evaluated CoQ₁₀ levels both in plasma and placenta among normal pregnant and PE primigravid women. Furthermore, as a highlight in design, the effect of high or low altitude residency was analyzed. Study found that women with PE (high or low altitude) displayed high placental CoQ₁₀ content, with significant lower plasma CoQ₁₀ levels among those residing in high altitude. The significance of presenting higher placental CoQ₁₀ level content in parallel with higher plasma levels is discussed.

Chapter 4 discusses the fact that endothelial dysfunction during PE is present in maternal and fetal vasculature. Two studies were designed to analyze this. *The first* measured NO levels in plasma and placenta of PE women and compared these to normal pregnant women. Again as a highlighted feature, effect of high or low altitude was analyzed. This study confirmed previous observations that women with PE displayed higher plasma and placental NO levels and that the differences were associated with altitude of residence. The effect of altitude is discussed. A *second study* further evaluated markers of endothelial function in the fetal circulation of women with PE. For this, NO, ADMA and VEGF levels were measured in the fetal circulation of pregnant women with severe PE. This study also performed genetic assessment. Indeed, DNA was extracted from umbilical vein to determine the frequency of VEGF gene single nucleotide polymorphisms. It is to highlight in this study that molecular analysis of endothelial function has been performed in women with PE to date only in the maternal side. Studies assessing the umbilical fetal circulation are scarce or lacking. Results found that women with severe PE displayed higher NO and ADMA fetal circulating levels (vein and artery) and lower VEGF umbilical vein levels. There was a significant trend of finding lower VEGF levels in the presence of -2578 CC and -1154 AG genotypes.

In **chapter 5**, the role of genetics in the pathogenesis of PE is explored with two interesting studies that assessed two single nucleotide polymorphisms of one candidate gene related to thrombophilia, the MTHFR gene (C667T and A1298C) in plasma (*the first study*) and in placenta (*second study*) of women complicated with PE.

The *first study* determined that the prevalence of the CC mutant genotype of the A1298C polymorphism was higher in plasma among PE women. This mutation among PE women was related to increased neck circumference and higher homocysteine (HCy) levels. No differences were found regarding the prevalence of the C677T polymorphisms among cases and control. To complement our first study we further analyzed in

a *second study* the prevalence of the same polymorphisms in placental material of PE cases compared to controls. Contrary to our previous study, frequency of the TT mutant genotype of the C677T polymorphism was higher in the placental material of pregnancies complicated with PE.

SUMMARY

To date the exact cause of PE is unknown. Despite this, studies have shown that PE is a multifactorial disease related to pregnancy which involves various pathways and mechanisms that are interconnected. The present thesis offers valuable candidates for both biomarkers for the evaluation of PE and for polymorphisms which might predispose to acquire this disease.

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CHAPTER 2

Vascular homeostasis affected during
pre- eclampsia:
increased anti-angiogenesis

Increased plasma soluble fms-like tyrosine kinase 1 and endoglin levels in pregnancies complicated with pre- eclampsia.

Chedraui P, Lockwood CJ, Schatz F, Buchwalder LF, Schwager G, Guerrero C, Escobar GS, Hidalgo L.

J Matern Fetal Neonatal Med 2009;22:565-70.

ABSTRACT

Background: Increased maternal plasma levels of proinflammatory cytokines as well as the anti-angiogenic agents soluble fms-like tyrosine kinase 1 (sFlt-1) and endoglin (sEng) are associated with promoting vascular dysfunction leading to the maternal syndrome of pre- eclampsia.

Objective and method: Nulliparous women complicated with pre- eclampsia ($n = 29$) and their corresponding controls ($n = 29$) delivering at the Enrique C. Sotomayor Obstetrics and Gynecology Hospital, Guayaquil-Ecuador were requested to participate in a study evaluating plasma levels of soluble anti-angiogenic factors (sFlt-1 and sEng) and pro-inflammatory cytokines: interleukin 6 (IL-6), interleukin 8 (IL-8), granulocyte colony stimulating factor (G-CSF), and tumor necrosis factor-alpha (TNF- α). Maternal and neonatal data were also assessed and compared among the study groups.

Results: No significant differences in either maternal baseline or delivery characteristics were observed among the study groups. Compared with controls, preeclamptic women exhibited higher plasma levels of sFlt-1 (19.0 ± 15.1 vs. 12 ± 8.3 ng/mL) and of sEng (20.4 ± 9.9 vs. 15.9 ± 9.4 ng/mL); respectively, $p < 0.05$. Women with severe disease displayed higher sFlt-1 and sEng levels when compared with mild ones (34.5 ± 11.6 vs. 9.5 ± 1.6 ng/mL, and 29.5 ± 9.0 vs. 14.8 ± 5.2 ng/mL, respectively; $p < 0.001$). In contrast, women with pre- eclampsia exhibited significant lower IL-8 and G-CSF levels compared with controls. No differences existed between either group in IL-6 levels or TNF- α .

Conclusion: Consistent with previous reports, increased sFlt-1 and Eng levels in maternal plasma is consistent with vascular dysfunction found in gestations complicated with pre- eclampsia.

Keywords: Pre- eclampsia, sFlt-1, endoglin, vascular dysfunction, cytokines

INTRODUCTION

Pre-eclampsia is the most common disorder of pregnancy complicating an estimated 7% of all pregnancies worldwide. Despite numerous studies and attempts at intervention, pre-eclampsia remains a leading cause of maternal and fetal morbidity and mortality in both developed and developing countries [1–5]. Reduced placental perfusion at early stages of pregnancy is a key event in the development of this disorder [6,7]. Defective trophoblastic invasion of the uterine spiral-arteries and arterioles leads to incomplete vascular remodeling and impaired utero-placental blood flow. Consequently, the placenta becomes ischemic and hypoxic and secretes into the maternal plasma an array of factors that are implicated in inducing generalized endothelial cell activation and vascular dysfunction that elicit the maternal syndrome [8,9]. Although the exact mechanisms involved remain unclear, vascular dysfunction found in pre-eclampsia is likely to be a consequence of reduced maternal circulating angiogenic factors [10–13] and increased levels of placental debris [14,15], reactive oxygen species [16,17], pro-inflammatory cytokines [tumor necrosis factor- α (TNF- α), interleukin 6 (IL-6) and interleukin 8 (IL-8)] [18–21] and anti-angiogenic factors [22–24].

Soluble receptor fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) are potent anti-angiogenic factors that are elevated in the plasma of women experiencing pre-eclampsia [25,26]. Increased levels of these factors are thought to contribute to maternal vascular dysfunction by binding to and thus reducing circulating levels of free vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) [27]. The aim of the present study was to compare levels of soluble anti-angiogenic factors and pro-inflammatory cytokines in the plasma obtained from women suffering with pre-eclampsia versus gestational matched control plasma samples.

MATERIALS AND METHODS

Participants and study design

This study represents a preliminary collaboration between the Institute of Biomedicine of the Medical Faculty, Universidad Católica, Guayaquil Ecuador (through its teaching facility the Enrique C. Sotomayor Gynecology and Obstetrics Hospital) and the Department of Obstetrics, Gynecology and Reproductive Sciences of Yale University School of Medicine, New Haven, Connecticut, USA. Sotomayor Hospital is a major referral center providing maternal/neonatal healthcare to the low income population of Guayaquil, Ecuador. It has the highest delivery rate in Latin America (>30,000 per year) concomitant with increased rates of high risk pregnancies [28–30]. For this study, nulliparous women with a singleton gestation fulfilling pre-eclampsia criteria admitted for delivery to the High Risk Pregnancy, Labor and Delivery Unit were recruited ($n = 29$). Normal

pregnant women ($n = 29$) delivering in the low risk Unit served as controls (matched for maternal and gestational age). All participants were recruited after signing informed consent of participation. Women with known medical disorders and on any medication before hospital admission (particularly non-steroidal anti-inflammatory drugs) were excluded from the study.

The diagnosis of pre-eclampsia depended on blood pressure $\geq 140/90$ mmHg on at least two occasions 6 h apart associated with proteinuria greater than ≥ 300 mg/dL as assessed by dipstick (>300 mg/dL) on two occasions 4–24 h apart [31]. Severity of pre-eclampsia was classified as mild or severe [32]. All preeclamptic women were previously normotensive at the time they became pregnant. Upon hospital admission, a 5 cc blood sample was taken from the antecubital vein of each subject and collected in tubes containing EDTA, which were centrifuged at 3000 rpm at 6°C for 20 min. The resulting plasma and cell layers were aliquoted into several micro centrifuge tubes, which were frozen and stored at -70°C until further analysis.

Maternal and neonatal data were recorded on a data sheet upon admission and included: maternal age, blood pressure and dipstick scores, neonatal gestational age and birthweight and route of delivery. The research study protocol was approved by the Institutional Review Board of the Enrique C. Sotomayor Hospital.

Biochemical assays

Commercial ELISA kits (Quantikine assays by R&D Systems, Minneapolis, MN) were used to measure immunoreactive levels of human sFlt-1, sEng and IL-6 in the plasma according to instructions provided by the manufacturer. The sFlt-1 assay has a sensitivity of 5.0 pg/mL, and intra-assay and interassay coefficients of variation of 3.3% and 7.6 %, respectively. The endoglin assay has a sensitivity of 0.007 ng/mL, and intra-assay and inter-assay coefficients of variation of 3.0% and 6.5%, respectively. The IL-6 assay has a sensitivity of 0.70 pg/mL, and intra-assay and inter-assay coefficients of variation of 2.6% and 4.5%, respectively.

A commercial multiplexable bead assay was used to measure the levels of immunoreactive human IL-8, G-CSF, and TNF- α (Bio-Plex cytokine panel; Bio-Rad Laboratories, Hercules, CA). The assay system for IL-8 has a sensitivity of 0.5 pg/mL, intraassay and inter-assay coefficients of variation of 4.0% and 9.1%, and percent recovery of 92%. The assay system for G-CSF has a sensitivity of 1.1 pg/mL, intra-assay and inter-assay coefficients of variation of 3.5% and 6.3%, and percent recovery of 99%. The assay system for TNF- α has a sensitivity of 3.0 pg/mL, intra-assay and inter-assay coefficients of variation of 5.0% and 9.4%, and percent recovery of 98%.

Statistical analysis

Statistical analysis was performed using SPSS software (SigmaStat version 3.0 for windows, Chicago Illinois, USA). Data are presented as mean + standard deviation (SD) and percentages (%). Comparison of means and percentages was performed using the Mann–Whitney *t*-test and the chi-square test, respectively. A *p*-value of <0.05 was considered to be statistically significant.

RESULTS

For this study, a total of 58 nulliparous women were recruited. These included 29 complicated with pre- eclampsia and 29 controls matched for maternal and gestational age. Among preeclamptic women, severe disease was observed in 17% (*n* = 5), moderate disease in 21% (*n* = 6) and mild disease in 62% (*n* = 18). Baseline and delivery characteristics as well as plasma concentrations of sFlt-1, sEng, IL-6, IL-8, G-CSF and TNF- α , of the studied subjects are depicted in Table I. No significant differences were found among the various groups in relation to maternal age, body mass index (BMI), income, educational level and delivery characteristics (neonatal gestational age, weight and cesarean section rate).

Compared with controls, matched for maternal and gestational age, preeclamptic women exhibited significantly higher plasma levels of sFlt-1 and sEng (Table I). Furthermore, within the preeclamptic group, the severe cases (*n* = 11) were significantly higher than the mild cases (*n* = 18) for levels of sFlt-1 (34.5 ± 11.6 vs. 9.5 ± 1.6 ng/mL, respectively; mean \pm SD, *p* < 0.001) and sEng (29.5 ± 9.0 vs. 14.8 ± 5.2 ng/mL, respectively; mean \pm S.D., *p* < 0.001) (Figure 1). In contrast, compared with controls, women with pre- eclampsia exhibited significantly lower plasma levels of IL-8 and of G-CSF levels (Table I). No significant differences between cases and controls were detected either in IL-6 or in TNF- α levels (Table I). Within the preeclamptic group, there were no significant differences between moderate-severe cases and mild cases for either IL-6, IL-8, G-CSF, or TNF- α .

DISCUSSION

Maternal–fetal interactions create a mild systemic inflammatory state exemplified by activation of both vascular endothelium and leukocytes that is most apparent in the third trimester of uncomplicated human pregnancies [33]. A further increase in systemic inflammation occurs in pre- eclampsia [33], which complicates about 7% of all pregnancies and is associated with a significantly increased risk for adverse maternal–fetal outcomes [1,5]. The initiation of most cases of pre- eclampsia has been related to shal-

low extravillous trophoblast invasion of the decidua and the resulting incomplete remodeling of the spiral arteries and arterioles [6,8]. However, the signs and symptoms of pre-eclampsia generally become apparent later in pregnancy usually in the third trimester [1] prompting significant investigation into identifying early biomarkers that could disclose those women that are likely to or even destined to develop pre-eclampsia.

Several studies have observed a positive correlation between the onset of systemic vascular dysfunction leading to the maternal syndrome of pre-eclampsia with a reciprocal reduction in circulating levels of the angiogenic factors, PLGF and VEGF [10–13] and increased levels of anti-angiogenic factors [26,34]. Elevated plasma anti-angiogenic factors (sFlt-1 and sEng) appear to bind to and lower VEGF and PLGF levels in the maternal plasma [26,34]. The resulting anti-angiogenic milieu is implicated in inducing systemic vascular damage, particularly as a target the kidney to induce hypertension and proteinuria of the maternal syndrome of pre-eclampsia [1].

Table I. Demographics, delivery characteristics and plasma analyte concentrations among studied women.

Variables	Pre-eclampsia (n = 29)	Control (n = 29)	p-value*
Baseline maternal characteristics			
Age (years)	20.4 ± 4.5 [†]	20.6 ± 4.2	NS
BMI (kg/m ²)	28.2 ± 3.3	27.4 ± 5.9	NS
Family income (\$US/month)	128.8 ± 43.1	168.0 ± 99.8	0.05
Educational level (years)	10.0 ± 3.9	9.9 ± 3.5	NS
Number of antenatal visits	6.6 ± 2.4	6.3 ± 2.2	NS
Systolic blood pressure (mmHg)	146 ± 10	109 ± 11	<0.001
Diastolic blood pressure (mmHg)	94 ± 9	71 ± 6	<0.001
Dipstick protein (range)	2 (1–3)	0 (0–0)	
Delivery characteristics			
Gestational age (weeks)	38.8 ± 1.1	38.6 ± 1.5	NS
Neonatal birth weight (g)	3,058 ± 427	2,859 ± 765	NS
Cesarean delivery (%)	18 (62)	16 (55.1)	NS
Plasma analyte concentration			
sFlt-1 (ng/mL)	19.0 ± 15.1	12.0 ± 8.3	0.020
Endoglin (ng/mL)	20.4 ± 9.9	15.9 ± 9.4	0.021
G-CSF (pg/mL)	30.9 ± 18.8	64.6 ± 49.5	0.015
IL-8 (pg/mL)	140.4 ± 112.6	180.1 ± 82.6	0.021
IL-6 (pg/mL)	20.8 ± 20.8	32.7 ± 32.7	NS
TNF-α (pg/mL)	65.6 ± 29.9	111.3 ± 173.2	NS

BMI, body mass index; sFlt-1, soluble fms-like tyrosine kinase 1; G-CSF, granulocyte colony stimulating factor; IL-8, interleukin-8; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha.

*Difference analyzed with Mann-Whitney *t*-test or chi-square test according to the case.

[†]Mean ± SD.

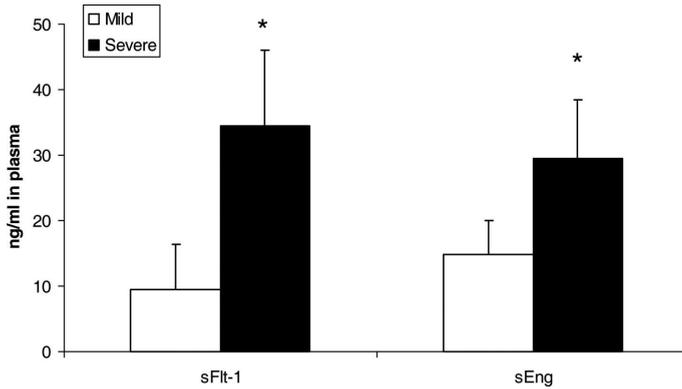


Figure 1. Comparison of sFlt-1 and sEng levels according to pre- eclampsia severity.

The increase in plasma levels of sFlt-1 and sEng in preeclamptic Ecuadorian women evaluated in the current study corresponds to reports in the literature for other populations of women that is consistent with the systemic anti-angiogenic milieu of pre- eclampsia [15,22,23]. However, unlike several reports indicating a pre- eclampsia-related elevation in plasma IL-8, G-CSF and IL-6 levels, the current study found that the plasma of preeclamptic Ecuadorian women contained lower IL-8 and G-CSF levels and similar IL-6 and TNF- α plasma levels compared with control women. These observations appear to contradict the general concept of pre- eclampsia as a systemic inflammatory state [33] since IL-8 and G-CSF are primarily involved in promoting acute inflammation via neutrophil recruitment and activation [35] and IL-6 helps to mediate the transition between acute and chronic inflammation via reciprocal inhibitory effects on neutrophils and stimulatory effects on monocytes/macrophages [36]. The solution to this apparent contradictory finding may lie in a fundamental difference between the patient population generally available in the USA and most European countries versus that recruited in the current study from Ecuador.

In developed countries such as the USA and most European countries, early diagnosis of the maternal syndrome prompts preterm delivery of the placenta to avoid maternal-fetal complications. In contrast, at Sotomayor Hospital, preeclamptic patients are more often seen and diagnosed at term. The resulting unfortunate delayed early detection of pre- eclampsia is directly related to the reality that 75.5% of women delivering at Sotomayor Hospital have inadequate prenatal care associated with higher parity, rural residency and general poverty conditions [29]. A direct consequence of this distinction is that published analytical results from preeclamptic and corresponding control plasmas tend to reflect preterm samples in developed countries and term samples in underdeveloped countries. The current results suggest for the first time that the antiangiogenic factors, but not pro-inflammatory cytokines, are maintained at elevated levels in preeclamptic plasma up through the time of delivery at term.

In summary, the current study confirms previous reports that the anti-angiogenic factors s-Flt1 and sEng are elevated in the plasma of preeclamptic women, and in those with moderate–severe disease. However, unlike several published studies, which have found elevated circulating levels of the proinflammatory cytokines G-CSF, IL-8, IL-6 in preeclamptic specimens, the current study detected lower levels of G-CSF and IL-8 and equivalent IL-6 and TNF- α levels in plasma of preeclamptic versus control women. These differential results likely reflect a fundamental difference between gestational age at the time of collection of the specimens (i.e. specimens derived from most published studies are pre-term, whereas those from the patient population of Sotomayor Hospital are term). Recent comparisons between preeclamptic and control patients from Sotomayor Hospital found significant differences between the groups in plasma nitric oxide levels [13] and in plasma levels and placental production of Co-enzyme Q₁₀ [12], which have been implicated in the pathogenesis of pre-eclampsia as abnormally secreted angiogenic factors [10,11].

Future studies aimed at obtaining complementary measurements of these endpoints as well as other pre-eclampsia-related biomarkers between the conventional patient and control populations in developed countries and those in underdeveloped countries as exemplified by patients from Sotomayor Hospital may well yield important information in elucidating mechanisms underlying the pathogenesis of pre-eclampsia.

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CHAPTER 3

In pre- eclampsia there is more to pathogenesis than vascular abnormalities: decreased anti-oxidation

Coenzyme Q₁₀ levels in women with pre- eclampsia living at different altitudes.

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ABSTRACT

Background: Pre- eclampsia is a common disorder of pregnancy exhibiting abnormal plasma and placental coenzyme Q₁₀ (CoQ₁₀) levels when compared to normal pregnancies.

Objective: To evaluate CoQ₁₀ levels both in plasma and placenta among normal pregnant ($n = 60$) and preeclamptic ($n = 63$) primigravid women and determine the effect of high or low altitude residency.

Study design: CoQ₁₀ was determined using High Performance Liquid Chromatography (HPLC) technique and group comparisons were performed.

Results: Preeclamptic women living at high altitude displayed significantly lower CoQ₁₀ plasma levels (0.64 ± 0.23 vs. 0.82 ± 0.46 $\mu\text{mol/L}$, $p = 0.05$). No differences were found in CoQ₁₀ plasma levels among women living at sea level. Interestingly, plasma CoQ₁₀ levels at low altitude in normal pregnancies were significantly lower than high altitude normal pregnancies. Compared to normal pregnancies, preeclamptic women displayed higher placental CoQ₁₀ content, which was only significant among those living at sea level (0.120 ± 0.07 vs. 0.076 ± 0.04 ng/mg protein , $p < 0.005$). Normal pregnant women living at high altitude displayed higher placental CoQ₁₀ content when compared to those residing at sea level ($p < 0.0005$).

Conclusion: Women suffering from pre- eclampsia (high or low altitude) display high placental CoQ₁₀ content, with significant low plasma CoQ₁₀ levels among those residing in high altitude. More research is warranted to establish the cause-effect relationship between CoQ₁₀ levels and pre- eclampsia.

Keywords: Coenzyme Q₁₀ , pre- eclampsia, pregnancy, placenta, altitude

INTRODUCTION

Pre-eclampsia is a common (~7% of all pregnancies) disorder of human pregnancy in which the normal hemodynamic response to pregnancy is compromised. It remains a leading cause of maternal morbidity and mortality, associated with a significant increase in perinatal mortality [3].

Clinical symptoms of pre-eclampsia arise from secondary systemic circulatory disturbances; there is evidence suggesting that its diverse manifestations, including altered vascular reactivity, vasospasm and discrete pathology in many organic systems, are derived from pathological changes within the maternal vascular endothelium [5]. Those changes, in addition to increased hypoxic conditions and oxidative stress, cause maternal symptoms of hypertension, proteinuria, clotting and liver dysfunction.

A key event in the development of pre-eclampsia is the reduction of placental perfusion in the early stages of pregnancy, which is due to defective trophoblastic invasion of uterine spiral-artery-vessel walls, therefore leading to poor development of the immature placenta and its maternal blood supply. This reduced placental perfusion leads to generalized dysfunction of the maternal vascular endothelium by mechanisms that still remain to be elucidated [18]. However, among patients with pre-eclampsia, there is an observed increase in the rate of lipid peroxidation, increased lipid availability, and decrease of several antioxidants such as alpha tocopherol, ascorbate, beta carotene and selenium [12]. Moreover, a significant decrease in plasma coenzyme Q 10 (CoQ₁₀) in women with pre-eclampsia has recently been reported [11,14].

Since CoQ₁₀ is a part of the non-enzymatic defense system against oxygen species (antioxidative function) [7], playing a key role in mitochondrial complexes I and III reaction mechanisms (electron transport) [1], it seems to be logical to hypothesize a role for CoQ₁₀ at the placental level. In this sense, we previously reported lower plasma and higher placental CoQ₁₀ content among preeclamptic women living in Quito, Ecuador (2,800 m above sea level) when compared to normal pregnancies [15]. On the other hand, it has recently been demonstrated that the number of uteroplacental vessels in low altitude placentas are significantly decreased compared to placentas at high altitude [16], a finding also reported in pre-eclampsia in which there were more uteroplacental arteries in placental bed biopsies from preeclamptic vs. uncomplicated pregnancies [13]. However, in normal pregnancies at sea level, significantly more arteries were remodeled per placenta than in normal pregnancies at high altitude [17]. Thus, the hypothesis that uteroplacental arterial remodeling would be reduced in high altitude pregnancy was supported, and the findings, as predicted, are intermediate between what is observed in pre-eclampsia and what is normal in healthy sea-level pregnancies [17]. Consistent with previous reports, uteroplacental arterial remodeling was not an 'all or none' phenomena at either high or low altitude [9], rather the proportion of arteries remodeled was reduced at high vs. moderate altitude.

Thus, it can be hypothesized that CoQ₁₀ content differs not only in pre-eclampsia, but also depends on women's altitude of residency. The aim of the present study was to evaluate the CoQ₁₀ levels both in plasma and placenta among normal pregnant and preeclamptic women residing either at high or low altitude.

PATIENTS AND METHODS

This study was carried out after approval of the Bioethics Committee of the Biomedical Center, Universidad Central, Quito, Ecuador, in which normal pregnant ($n = 60$) and preeclamptic ($n = 63$) primigravid women admitted to the "Hospital Gineco Obstétrico Isidro Ayora" in Quito, Ecuador and the "Hospital Gineco Obstétrico Enrique C. Sotomayor" in Guayaquil, Ecuador, were recruited after signing informed consent of participation. Out of a total of 123 pregnant women, 63 resided in Quito (2,800 m altitude) and 60 in Guayaquil (sea level). Women with a medical history of cardiovascular or gynecological problems, and on any medication (especially non steroidal anti-inflammatory drugs) were excluded from the study. Women with pre-eclampsia registered blood pressures over 140/90 mmHg on at least two occasions 6 hours apart, and proteinuria greater than ++ as assessed by dipstick (>300 mg/dL) on two occasions 4 to 24 hours apart. Blood pressure was measured as previously described [8]. For the measurement of CoQ₁₀, a blood sample (10 ml) was obtained from each woman at the antecubital venous puncture site, and immediately transferred into a polypropylene vial containing 3.15% sodium citrate (1:9, v/v). These samples were centrifuged at room temperature and 500 μ l plasma aliquots were frozen at -40°C . After delivery of the placenta, a sample (2–3 g) from the maternal side was obtained (any infarcted area was avoided), washed twice with saline solution to remove the excess of blood, and transferred into a vial containing Krebs buffer solution. Measurement of CoQ₁₀ content in placenta was carried out with approximately 100 mg of freeze-clamped tissue accurately weighed in the frozen state and subsequently homogenized with 2 ml of Krebs buffer in a glass homogenizer with a manual pestle, as described elsewhere [6]. Briefly, prior to homogenization, 50 μ l of ethanolic BHT (2,6-di-tert-butyl-p-cresol; Sigma Aldrich, MO, USA) was added to prevent lipid autoxidation without reducing the ubiquinones. After addition of 1 ml of 0.1 M aqueous sodium dodecyl sulphate (SDS; Sigma-Aldrich, MO, USA) and a brief mixing by homogenization, the sample was transferred to a 10 ml test tube, fitted with a Teflon-lined screw cap. The homogenizer was rinsed with 2 ml of reagent alcohol, which was combined with the homogenate. The mixture was vortexed for 30 s, and 2 ml of hexane (Merck, Darmstadt, Germany) was added, and the tightly screwed test tube was vigorously vortexed for 2 min. It was then centrifuged for 5 min at 1,000 g to separate the layers. One milliliter of the hexane layer was transferred to a small vial and dried under nitrogen. The residue was redissolved in methanol/[ethanol/isopropanol 95/5, v/v] (1:1 v/v). Samples were analyzed immediately and

kept on ice and covered with aluminum foil to prevent photodegradation of ubiquinones. CoQ₁₀ was measured in a high performance liquid chromatography (HPLC) system (Perkin-Elmer, CN, USA) equipped with a Lichrosorb® RP18 (5 μm, 125 × 4 mm; Phenomenex, CA, USA) column and with a guard column (Merck, Darmstadt, Germany), as previously described [14]. Samples were measured in duplicates and the mean value was used for statistical analysis.

Data was analyzed using GraphPad InStat version 3.01 for Windows (GraphPad Software, CA) and presented as mean ± standard deviations (S.D). Comparison of continuous data was performed with unpaired student T test. A *p* value of < 0.05 was considered as statistically significant.

RESULTS

Characteristics of women and neonates included in the study are presented in Table 1. Placental and neonatal weight at delivery from women with pre- eclampsia living in high altitude were found to be significantly lower. Preeclamptic women living at high altitude showed significantly lower CoQ₁₀ plasma levels (0.64 ± 0.23 vs. 0.82 ± 0.46 μmol/L, *p* = 0.05). Contrarily, there were no differences in plasma CoQ₁₀ levels among women living at sea level (normal: 0.50 ± 0.18 vs. pre- eclampsia: 0.51 ± 0.19 μmol/L, *p* = *NS*). Interestingly, plasma levels of CoQ₁₀ in normal pregnant women living at sea level were significantly lower than those in normal pregnant women living at high altitude (*p* = 0.001) and comparable to those found in pre- eclampsia at high altitude (*p* = *NS*, Fig. 1). Placental CoQ₁₀ content was found to be significantly higher among preeclamptic women living at sea level compared to those with normal pregnancies (0.120 ± 0.07 vs. 0.076 ± 0.04 ng/mg protein, *p* < 0.005). The same trend was found among women living at high altitude although this was not found to be significant (0.159 ± 0.13 vs. 0.135 ± 0.07 ng/mg protein, *p* = *NS*). Although placental CoQ₁₀ content was found to be significantly higher among normal pregnant women at high altitude compared to those residing at sea level (*p* < 0.0005), no differences were found among preeclamptic women, either at high altitude or sea level (Fig. 2).

DISCUSSION

Despite the limitations of this study (cross-sectional design and small sample size) it is the first to determine CoQ₁₀ levels, both in plasma and placenta, simultaneously in pregnant women residing either at sea level or at high altitude. In preeclamptic women residing at high altitude, there were lower CoQ₁₀ plasma levels when compared to normal pregnancies. However, it might be important to emphasize that this difference was still present, although plasma levels in normal pregnant women residing at high altitude

in the present report were slightly lower than those previously reported [15]. It is possible that gestational age at which samples were taken was responsible for this difference (unpublished data). In the present series, there were unexpected findings, not only the absence of differences in CoQ₁₀ plasma levels between normal pregnant and preeclamptic women living at sea level; also for the relatively low CoQ₁₀ plasma levels found in normal pregnant women residing at sea level. The latest results, that is in clear discrepancy with previous reports in pregnant women living close to the sea level [10,11], could not be interpreted without having the CoQ₁₀ plasma levels in a group of Ecuadorian non-pregnant women residing at sea level, an additional control group not planned in this study. Contrarily to plasma levels, placental CoQ₁₀ content appeared to be higher in preeclamptic women as compared to normal pregnancies, independently of the altitude of women's residency. This might be a surprising finding, as plasma concentrations of CoQ₁₀ are significantly influenced by dietary uptake [19], while tissue levels of CoQ₁₀ depend mainly on de novo synthesis [20]. Therefore, dietary CoQ₁₀ concentrations could mask tissue deficiencies. On the other hand, it has been suggested that higher tissue concentrations of ubiquinone may be a result of a higher metabolic rate [2]. In this sense, higher placental CoQ₁₀ contents were found among sea level residing Italian pregnant women with HELLP syndrome (a severe complication of pre-eclampsia) when compared to normal pregnancies, but plasma levels were not measured [4].

Table 1: Characteristics of gravids and neonates included in the study

Parameter			Quito		Guayaquil	
	Normal n = 60	PE n = 63	Normal n = 30	PE n = 33	Normal n = 30	PE n = 30
Maternal age (years)	20.7 ± 4.4 ^a	20.6 ± 4.4	20.6 ± 4.6	20.6 ± 4.4	20.7 ± 4.2	20.6 ± 4.5
Systolic blood pressure (mmHg)	110.2 ± 9.3	144.9 ± 11 ^b	110.7 ± 6.8	143.6 ± 11.4 ^b	109.7 ± 11.3	146.3 ± 10.7 ^b
Diastolic blood pressure (mmHg)	96.8 ± 10.5	72.1 ± 6.6 ^b	73 ± 7	98.9 ± 7 ^b	71.3 ± 6.2	94.5 ± 9.7 ^b
Neonatal gestational age at delivery (weeks)	38.5 ± 1.6	38.6 ± 1.3	38.7 ± 1.1	38.2 ± 1.9	38.6 ± 1.5	38.8 ± 1
Neonatal weight at delivery (grs)	2,980.8 ± 597.8	2,908.8 ± 454.4	3,097 ± 364.7	2,757.8 ± 429.1 ^b	2,864 ± 752.2	3,073 ± 429.1 ^c
Placental weight (grs)	576.4 ± 98.5	556 ± 130.8	580.1 ± 98.5	503.5 ± 120.1 ^b	572.6 ± 100	614 ± 118 ^c

^a Mean ± standard deviation; ^b $p < 0.05$ compared to normal gestations (non paired t student's test); PE: pre-eclampsia; ^c $p < 0.05$ compared to PE or normal gestations from Quito.

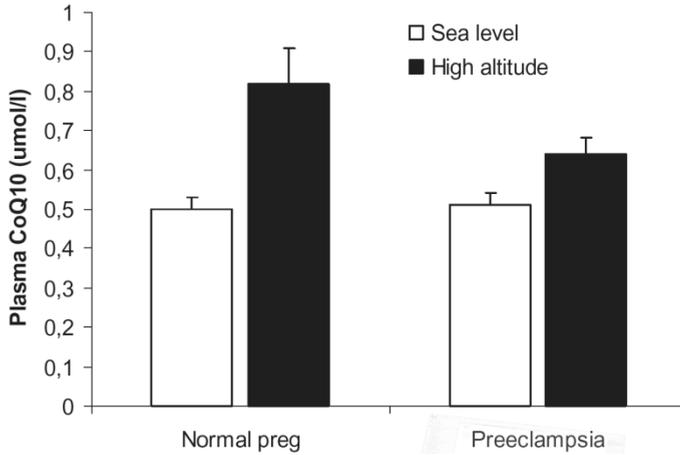


Fig. 1. Coenzyme Q₁₀ plasma levels in normal pregnancies and during pre- eclampsia according to their residency (sea level or high altitude).

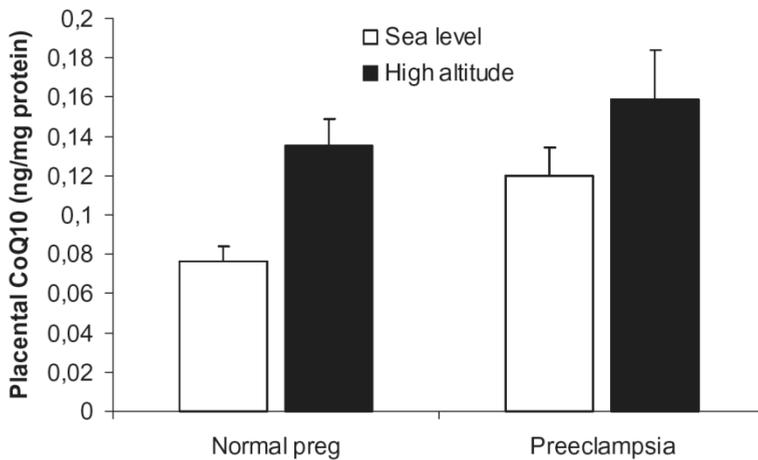


Fig. 2. Coenzyme Q₁₀ placental content in normal pregnancies and during pre- eclampsia according to their residency (sea level and high altitude).

The observations in our study allow us to hypothesize that CoQ₁₀ plasma levels are not directly related to placental CoQ₁₀ content. Moreover, it is also plausible to assume that placental development is totally an energy dependent process and that CoQ₁₀ could be an essential element in its physiological role. If this is true, then the next obvious questions to be considered should be: what occurs during pre- eclampsia? Are these high placental levels a late compensatory mechanism?

In conclusion it was determined that (1) during pregnancy there is no relationship between plasma CoQ₁₀ levels and placental CoQ₁₀ content and (2) women complicated with pre- eclampsia (high or low altitude) displayed high placental CoQ₁₀ content, with

significantly low plasma CoQ₁₀ levels among those residing at high altitude. More research is warranted to establish the cause-effect relationship between CoQ₁₀ levels and pre-eclampsia.

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CHAPTER 4

Endothelial dysfunction during pre- eclampsia is present in the maternal and fetal vasculature

CHAPTER 4A

Plasma and placental nitric oxide levels in women with and without pre-eclampsia living at different altitudes.

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Int J Gynaecol Obstet 2009;104:140-2.

ABSTRACT

Objective: To investigate the nitric oxide (NO) levels in the plasma and the placentas of pregnant women with pre-eclampsia and women without pre-eclampsia, and to determine the effect of high or low altitude of residence.

Methods: NO was determined by chemoluminescence and group comparisons were performed.

Results: Women with pre-eclampsia ($n = 63$) had higher plasma NO levels (38.6 ± 17.44 vs 30.6 ± 12.44 $\mu\text{mol/L}$, $P = 0.004$) and higher placental NO levels (38.5 ± 17.0 vs 24.3 ± 7.16 ng/mg protein, $P < 0.05$) compared with women without pre-eclampsia. A similar trend was found when comparisons were made according to altitude of residence. NO levels were significantly higher in the plasma of pre-eclamptic women living at sea level (41.11 ± 18.78 vs 28.96 ± 9.57 $\mu\text{mol/L}$, $P = 0.003$), and in the placentas of women living at high altitude (39.51 ± 16.98 vs 21.91 ± 6.64 ng/mg protein, $P < 0.0001$).

Conclusion: Women with pre-eclampsia had higher plasma and placental NO levels and the differences were associated with altitude of residence.

Keywords: Altitude, Nitric Oxide, Pre-eclampsia, Placenta, Pregnancy

INTRODUCTION

Pre-eclampsia is a common disorder of human pregnancy (affecting about 7% of all pregnancies), in which the normal hemodynamic response is compromised. It remains a leading cause of maternal and fetal morbidity and mortality [1]. Reduced placental perfusion in the early stages of pregnancy is a key event in the development of this disorder. Defective trophoblastic invasion of the uterine spiral artery vessel walls leads to poor placental development (decreased maternal blood supply), which ultimately produces generalized vascular endothelial dysfunction by mechanisms that remain to be elucidated [2].

Several factors may be important in the physiological mechanisms of placental angiogenesis and regulation of vascular tone, and these factors could have major roles in the pathogenesis of abnormal placental functioning. One of these factors is nitric oxide (NO), the intracellular gaseous messenger synthesized by NO synthase (NOS) from L-arginine and oxygen. NO exerts diverse biological functions in several physiological and pathological processes, especially in vascular pathophysiology [3]. NO produced by syncytiotrophoblast-derived endothelial cells is thought to cause dilation of the human placental vasculature [4], and may act as a paracrine agent for the maintenance of uterine quiescence during pregnancy. Additionally, local placental NO generation may be essential to promote cytotrophoblast endovascular invasion, an essential feature of normal placentation [5].

During ischemia–reperfusion and exposure to high altitude, a change in the balance of the levels of reactive oxygen species (ROS) and NO occurs [6]. In contrast to ischemia–reperfusion, ROS levels increase during hypoxia, returning to pre-hypoxic values after re-establishing normoxia. Hence, hypoxia leads to an alteration of the ROS–NO balance, which is eventually restored during the acclimatization process through the up-regulation of inducible NOS (iNOS) [7]. The findings of Serrano et al. [8], in a study of the rat cerebellum after reoxygenation at sea level, indicate the involvement of a different type of NOS, which produces dissimilar NO production at high altitude and can lead to an increased formation of nitrotyrosine. In addition, there may be beneficial effects at the tissue level [9] with increased numbers of mitochondria and enhanced function resulting from higher levels of NO [10]. However, data derived from tissue hypoxia–reperfusion models suggest that excess NO may be detrimental [10], specifically in the production of additional oxidative and free radical-mediated tissue damage. To the best of our knowledge, studies measuring placental NO levels, either at sea level or at altitude, are lacking. Thus, it can be hypothesized that NO levels differ not only in pre-eclampsia, but are also dependent on the women's altitude of residence. The aim of the present study was to evaluate NO levels in the plasma and placentas of pregnant women with and without pre-eclampsia residing at high or low altitude.

MATERIALS AND METHODS

The study was carried out with approval granted by the Bioethics Committee of the Biomedical Center in Quito, Ecuador. Primigravid pregnant women without pre-eclampsia ($n = 60$) and those with pre-eclampsia ($n = 63$), who were admitted to the Hospital Gineco-Obstetrico Isidro Ayora (Quito, Ecuador) and the Hospital Gineco-Obstetrico Enrique C. Sotomayor (Guayaquil, Ecuador), were recruited after signing an informed consent form. Of the 123 pregnant women enrolled, 63 resided in Quito (2800 m altitude) and 60 in Guayaquil (sea level). Women with known medical disorders and those receiving medication before hospital admission (especially non-steroidal anti-inflammatory drugs) were excluded from the study. Women with pre-eclampsia had blood pressures greater than 140/90 mm Hg on at least 2 occasions 6 hours apart, and proteinuria levels producing a 2+ reaction on a standard urine dipstick (> 300 mg/dL) on 2 occasions 4–24 hours apart. Blood pressure was measured as previously described [11]. Upon hospital admission a 10-mL blood sample was taken from each participant from the antecubital vein, and the sample was immediately transferred into a polypropylene vial containing 3.15% sodium citrate (1:9 v/v). Samples were centrifuged at room temperature and the 500 μ L plasma aliquots were frozen at -40 °C. After delivery of the placenta, a 2–3-g sample from the maternal side was obtained (any infarcted area was avoided), washed twice with saline solution to remove excess blood, and transferred into a vial containing Krebs buffer solution. Measurement of the placental NO was performed using approximately 100 mg of freeze-clamped tissue accurately weighed in the frozen state, and subsequently homogenized in a glass homogenizer with 2 mL of Krebs buffer and a pestle. The supernatant was used for the measurements. Plasma and supernatant NO values were determined by a chemoluminescence technique (Sievers NOA-280; Sievers, Boulder, CO, USA) as previously described [12]. Samples were measured in duplicate and mean values were used for statistical analysis.

Data were analyzed using GraphPad InStat version 3.01 for Windows (GraphPad Software, CA, USA) and presented as means \pm SD. Comparison of continuous data was performed using the non-parametric Mann-Whitney test. $P < 0.05$ was considered statistically significant.

RESULTS

The characteristics of women and neonates included in the study are presented in Table 1. Of the women with pre-eclampsia ($n = 63$), 47.6% had severe disease and there was no statistical difference found between those living at high altitude (54.5%) or at sea level (40%).

Placental and neonatal weights of the pre-eclamptic women delivering at high altitude were significantly lower than women who did not have pre-eclampsia living at the

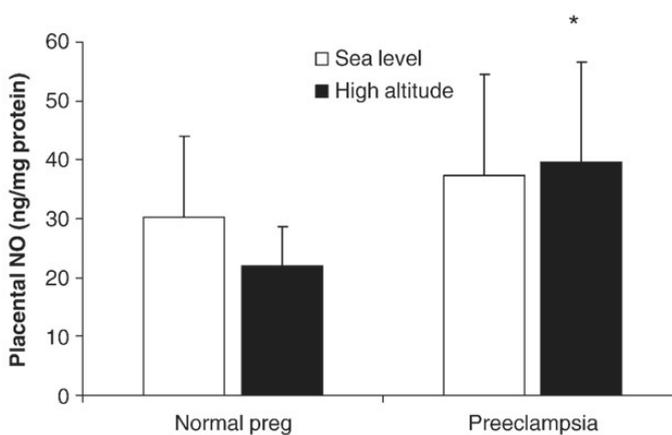
same altitude. Women with pre eclampsia ($n = 63$) had higher plasma and placental NO levels compared with women without pre-eclampsia (38.6 ± 17.44 vs 30.6 ± 12.44 $\mu\text{mol/L}$, $P = 0.004$; and 38.5 ± 17.0 vs 24.3 ± 7.16 ng/mg of protein, $P < 0.05$). A similar trend was found once distributed by altitude of residence, with significantly higher NO levels found in the plasma of pre-eclamptic women living at sea level (41.11 ± 18.78 vs 28.96 ± 9.57 $\mu\text{mol/L}$, $P = 0.003$; Table 1) and in the placentas of women living at high altitude (39.51 ± 16.98 vs 21.91 ± 6.64 ng/mg of protein, $P < 0.0001$; Fig. 1).

Table 1 Characteristics of neonates and pregnant women with pre-eclampsia and those without pre-eclampsia living at high altitude and at sea level^a

Parameter			High altitude		Sea level	
	Without PE (n = 60)	With PE (n = 63)	Without PE (n = 30)	With PE (n = 33)	Without PE (n = 30)	With PE (n = 30)
Maternal age, y	20.7 ± 4.4	20.6 ± 4.4	20.6 ± 4.6	20.6 ± 4.4	20.7 ± 4.2	20.6 ± 4.5
Gestational age, wk	39 ± 1.1	38.2 ± 1.9	39 ± 1.1	38.2 ± 1.9	38.6 ± 1.5	39.1 ± 1.0
Systolic blood pressure, mm Hg	110.2 ± 9.3	144.9 ± 11 ^b	110.7 ± 6.8	143.6 ± 11.4 ^b	109.7 ± 11.3	146.3 ± 10.7 ^b
Diastolic blood pressure, mm Hg	72.1 ± 6.6	96.8 ± 10.5 ^b	73 ± 7.0	98.9 ± 7 b	71.3 ± 6.2	94.5 ± 9.7
Neonatal gestational age at delivery, wk	38.5 ± 1.6	38.6 ± 1.3	38.7 ± 1.1	38.2 ± 1.9	38.6 ± 1.5	38.8 ± 1.0
Neonatal weight at delivery, g	2980.8 ± 597.8	2908.8 ± 454.4	3097 ± 364.7	2757.8 ± 429.1 ^b	2864 ± 752.2	3073 ± 429.1 ^c
Placental weight, g	576.4 ± 98.5	556 ± 130.8	580.1 ± 98.5	503.5 ± 120.1 ^b	572.6 ± 100	614 ± 118 ^c
Plasma nitric oxide, $\mu\text{mol/L}$	30.6 ± 12.44	38.6 ± 17.44 ^b	34.3 ± 14.11	38.21 ± 14.97	28.96 ± 9.57	41.11 ± 18.78 ^b
Placental nitric oxide, ng/mg of protein	24.3 ± 7.16	38.5 ± 17.0 b	21.91 ± 6.64	39.51 ± 16.98 ^b	30.33 ± 13.65 ^c	37.34 ± 17.15

Abbreviation: PE, pre-eclampsia.

^a Values are given as mean ± SD. ^b $P < 0.05$ comparing women without pre-eclampsia. ^c $P < 0.05$ comparing women with pre-eclampsia or without pre-eclampsia at high altitude.



* $P < 0.0001$ comparing pregnant women without pre-eclampsia at high altitude.

Fig. 1. NO content in placental tissue from pregnant women without pre-eclampsia and pregnant women with pre-eclampsia residing at high altitude and at sea level.

DISCUSSION

The results of this study showed that compared with pregnant women without pre-eclampsia, women with pre-eclampsia exhibit significantly higher plasma NO levels. Although this is in agreement with our previous observations [12–15], the reported levels of NO in relation to pre-eclampsia are contradictory [16], which is probably due to differences in the measurement methods and/or the metabolite analyzed. Nevertheless, there is agreement that pre-eclampsia is associated with altered NO production and/or activity.

Moreover, plasma and placental NO measurements were performed in women residing at sea level and at high altitude. The NO level was found to be higher in the placentas of women with pre-eclampsia ($n = 63$), and was more pronounced among women living at high altitude, and interestingly, these women also had neonates and placentas of a lower weight compared with those of pre-eclamptic women living at sea level.

The expression of iNOS and endothelial nitric oxide synthase (eNOS) has been previously reported to be significantly lower in the placentas of growth-restricted fetuses [17], but not in placentas from women with pre-eclampsia. Fetal–placental NOS activity and the NO concentration in the umbilical circulation have been found to be altered in pregnancies complicated by pre-eclampsia [17]. One study of women with pre-eclampsia specifically described a decreased or unchanged placental NOS activity [18]. NO in the fetal–placental circulation is derived from eNOS activity, found predominantly in the syncytiotrophoblast [19]. Overall, it is possible to conclude that plasma NO activity is altered in women with pre-eclampsia. It is also altered in the placenta, ie, the NO levels were higher in the placentas of women with pre-eclampsia. However, a recent study demonstrated that NOS knockout mice do not develop hypertension during pregnancy, perhaps due to the possible recruitment of compensatory vasodilator substances (eg, prostacyclin) [20]. Moreover, in placentas from women with pre-eclampsia, decreased antioxidant capacities [21–23] and abnormally high rates of superoxide synthesis have been reported [24]. Therefore, based on the finding that women with pre-eclampsia living at high altitude had higher placental NO levels and associated lower placental and neonatal weights, compared with those living at sea level, we hypothesize that the placental NO is not fully utilized and could react with a ROS (ie, superoxide), to produce deleterious effects. This hypothesis could be supported by other important changes in the mechanisms regulating NOS activation and vasodilatation found during pre-eclampsia. Together with other contributing factors, such as angiotensin II receptor upregulation, angiotensin receptor agonistic auto-antibodies, and endothelin-1 upregulation [3], this could trigger placental oxidative stress and contribute to vascular resistance dysregulation resulting in increased sensitivity to vasopressor agents.

Despite the limitations of this study, namely its cross-sectional design and small sample size, women with pre-eclampsia were found to have higher plasma and placen-

tal NO levels, with significant differences found depending upon the altitude of residence. More research in this area is required.

Acknowledgments

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CHAPTER 4B

Feto-placental nitric oxide, asymmetric dimethylarginine and vascular endothelial growth factor (VEGF) levels and VEGF gene polymorphisms in severe pre- eclampsia

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ABSTRACT

Objective: To measure plasma nitric oxide (NO), asymmetric dimethylarginine (ADMA) and vascular endothelial growth factor (VEGF) levels and VEGF gene polymorphisms in fetal circulation in severe pre- eclampsia.

Methods: Cord vessels of singleton gestations complicated with severe pre- eclampsia 36 weeks or more ($n = 31$) and controls were sampled upon delivery for analyte measuring. Additionally, DNA was extracted from umbilical vein whole blood to determine the frequency of VEGF gene single nucleotide polymorphisms (SNPs): -2578 A/C, -1498 C/T, -1154 A/G, -634 C/G and $+936$ C/T. Coefficient correlations between analyte levels and placental and neonatal weight were calculated.

Results: NO plasma levels in umbilical vessels (artery and vein) were significantly higher in pre- eclampsia cases as compared to controls (4.67 ± 3.0 vs. 0.82 ± 0.90 ; 4.46 ± 3.0 vs. 0.82 ± 0.99 mmol/L, respectively, $p = 0.0001$ both). ADMA levels displayed a similar increased trend in both fetal vessels, but this did not reach statistical significance (2.57 ± 1.03 vs. 2.34 ± 0.57 ; 2.74 ± 0.94 vs. 2.42 ± 0.59 mmol/L, respectively, $p > 0.05$). VEGF was significantly lower in artery but not in vein in pre- eclampsia cases (200.48 ± 225.62 vs. 338.61 ± 287.03 pg/mL, $p = 0.04$). A significant positive correlation was found between NO and ADMA levels (artery and vein) among pre- eclampsia cases. Overall, the frequency of the studied VEGF gene SNPs did not differ among pre-eclamptic cases and controls; nevertheless, a significant trend toward lower umbilical vein VEGF levels was observed in pre-eclampsia cases in the presence of -2578 CC and -1154 AG genotypes.

Conclusion: Near term gestations complicated with severe pre- eclampsia presented higher NO levels in fetal circulation, which correlated to ADMA and lower artery VEGF values. More research is warranted to confirm that selected VEGF SNPs may be associated with lower umbilical vein VEGF.

Keywords: Asymmetric dimethylarginine, nitric oxide, pre- eclampsia, umbilical vessels, vascular endothelial growth factor

INTRODUCTION

Despite attempts at intervention, pre-eclampsia is still a leading cause of maternal and fetal morbidity and mortality [1]. Reduced placental perfusion at early stages of pregnancy is a key event in its development [2], in which defective trophoblastic invasion of the uterine spiral-arteries and arterioles leads to incomplete vascular remodeling and impaired utero-placental blood flow. Although the intrinsic involved mechanisms are still unclear, vascular dysfunction found in pre-eclampsia is likely to be a consequence of reduced maternal circulating angiogenic factors [3] and increased levels of placental debris [4], reactive oxygen species [5], pro-inflammatory cytokines [6], and anti-angiogenic factors [7]. Nitric oxide (NO) synthesis is involved in some of these proposed pathological mechanisms. However, how NO exerts its effects over vascular development and placental function in pre-eclampsia is still unclear [8]. In pre-eclampsia, increased maternal and fetal serum NO levels may be a compensatory protective mechanism to maintain blood flow and reduce platelet aggregation in the fetal and maternal circulation. In addition, NO production is directly related to pre-eclampsia severity [9]. Asymmetric dimethylarginine (ADMA) is an aminoacid that circulates in plasma, is excreted in urine, and is found in various tissues and cells. It inhibits NO synthase (NOS) and has been proposed as a marker of endothelial dysfunction. ADMA is increased in women with pre-eclampsia, even before clinical manifestation [10,11]. On the other hand, vascular endothelial growth factor (VEGF) mediates important signaling pathways during fetal growth and in the maternal circulatory system. VEGF concentration is deranged in gestations complicated with pre-eclampsia [12].

Genetic background may also influence pre-eclampsia development and the concomitant endothelial dysfunction. Hypertension and angiogenesis are linked, since microcirculatory vasoconstriction is in part due to defective angiogenic processes [13]. Single nucleotide polymorphisms (SNPs) of the promoter region of the VEGF gene are related to higher or lower VEGF production and to altered risk of developing diseases characterized by deranged angiogenesis [14]. In particular, the VEGF -2578 CC genotype has been associated with higher VEGF expression than the AA genotype, which is consistent for a protective effect of VEGF in atherosclerosis development [15]. Recent studies suggest that some VEGF genotypes are more common in women with pre-eclampsia [16]. However, it is currently unknown whether such genetic asset is related to abnormal circulating amounts of VEGF during pre-eclampsia.

The aim of the present study was to measure plasma NO, ADMA, and VEGF levels in fetal circulation in gestations complicated with severe pre-eclampsia. Additionally, the frequency of common SNPs of the VEGF gene and their relation to circulating umbilical vein VEGF levels were also analyzed.

MATERIALS AND METHODS

Study design and population

The present pilot research collaboration was carried out at the Enrique C. Sotomayor Obstetrics and Gynecology Hospital, Guayaquil, Ecuador. Sotomayor Hospital is a major referral center that provides maternal and neonatal healthcare to the low-income population of Guayaquil, Ecuador. It has the highest delivery rate in Latin America (>30 000 per year) in addition to increased rates of high risk pregnancies [17].

For this study, singleton gestations fulfilling severe pre- eclampsia criteria admitted for delivery to the High Risk Pregnancy Labor and Delivery Unit were recruited. Normal pregnant women delivering in the low risk Unit served as controls which were matched for maternal and gestational age, parity, and laboring status. All participants were recruited after signing informed consent. Women with known medical disorders and on any medication before hospital admission (particularly non-steroidal anti-inflammatory drugs) were excluded from the study.

ACOG criteria was used to define severe pre- eclampsia as a blood pressure \geq 160/110 mmHg on two occasions at least 6 hours apart and proteinuria +++ or more on at least two random samples 4 hours apart [18].

Maternal and neonatal information was recorded on a data sheet and included: maternal age, blood pressure and dipstick scores, neonatal gestational age and birth weight and route of delivery. The Institutional Review Board of the Enrique C. Sotomayor Hospital approved the research study protocol.

Biochemical assays

Blood samples

Upon infant delivery a 25 cm fetal cord segment was double clamped from which a 5 ml blood sample was taken from each cord vessel (artery and vein). Tubes were immediately centrifuged at 3,000 rpm at 6°C for 20 minutes. The plasma and cell layers were aliquoted into several micro-centrifuge tubes, which were frozen and stored at -70°C until further analysis.

Nitrite assay

Nitric oxide production was determined by a nitrite assay using 2, 3-diaminonaphthalene. Fluorescence of 1-(H)-naphthotriazole was measured by excitation and emission wavelengths of 365 and 450 nm. Standard curves were constructed with sodium nitrite. A stock solution of 1 mmol NO_3 in PBS (pH 7.4) was prepared and then standards (from 0 to 50 $\mu\text{M/L}$) were obtained by diluting the stock solution in PBS solution (pH 7.4). Plasma, blank, standard, and control samples (250 μl each) were diluted by adding 250 μl PBS to plastic vials. Each mixture was then thoroughly mixed and the

plasma centrifuged in spin filter columns for 60 minutes at room temperature. Subsequently, 100 µl Griess reagent, 1% sulphanilamide + 0.1% naphthylethylenediamine dihydrochloride prepared in 3.1% H₃PO₄ solution was added to each vial. Reagents were mixed on a shaker for one minute at 185 rpm and plates incubated for 10 minutes at 37°C. Samples were placed in a spectrophotometer and the absorbance was read at 490 nm. Non-specific fluorescence was determined in the presence of NG-monomethyl-L-arginine (3 mmol/L). All samples, kit controls and standards were analyzed in duplicates.

Enzyme-linked immunosorbent assay

Acylation was conducted in the 96-well reaction plate supplied with the kit according to the instructions of the manufacturer. Standards, kit controls and samples (20 µl) were mixed with 25 µl acylation buffer and 25 µl equalizing reagent. Subsequently, 25 µl of acylation reagent was added and the reaction plate incubated for 30 minutes at room temperature on an orbital shaker. Diluted equalizing reagent (100 µl) was added and incubation continued for 45 minutes. After incubation, samples were ready for the enzyme-linked immunosorbent assay (ELISA) analysis.

The ADMA-ELISA kit (Biovendor, Cat. No.: REA201/96) consists of a split-type reaction plate (12 × 8) coated with ADMA, six standards (0–5 µM), rabbit anti-ADMA antiserum, goat anti-rabbit-IgG-peroxidase conjugate, TMB substrate solution, stop solution, and wash buffer. Aliquots (50 µl) of the acylated standards, kit controls or samples were processed according to the instructions of the kit manufacturer. Absorbances were measured with a microplate reader using a wavelength of 450 nm (reference wavelength 620 nm). All samples, kit controls, and standards were analyzed in duplicates.

The VEGF-A-ELISA kit (Antigenix America, Cat. No. RH88820CKC) consists of a split-type reaction plate (12 × 8) coated with VEGF-A polyclonal antibodies, goat anti-VEGF-A antiserum, anti-goat- Biotin-Conjugate, TMB substrate solution, stop solution, streptavidin-HRP, and wash buffer. Absorbances were measured with a microplate reader using a wavelength of 450 nm (reference wavelength 620 nm). All samples, kit controls, and standards were analyzed in duplicates.

VEGF genotyping

DNA was extracted from whole blood of the umbilical vein using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Genotyping was performed using the Taqman platform using specific and validated primers (Applied Biosystems, Carlsbad, CA, USA) with an ABI PRISM 7900HT Sequence Detection System. The investigated SNPs were the following: -2578 A/C (rs699947; TaqMan SNP genotyping assay C_8311602_10), -1498 C/T (rs833061; C_1647381_10), -1154 A/G (rs1570360; C_1647379_10), -634 C/G (rs2010963; C_8311614_10), +936 C/T (rs3025039; C_16198794_10). PCR reaction was carried out according to the protocol of the manufacturer. Allelic distribution for VEGF SNPs was in Hardy-Weinberg equilibrium.

Statistical analysis

Statistical analysis was performed using SPSS statistical package (Version 19.0 for Windows, SPSS Inc, Chicago, IL, USA). Data are presented as means, standard deviations, medians, interquartile ranges, coefficients, and percentages. The Kolmogorov Smirnov test was used to assess the normality of data distribution. According to this, continuous data were compared with student's *t*-test (parametric), the Mann–Whitney test (non-parametric) or the Kruskal–Wallis test (various non-parametric samples). Chi-square, Yates' corrected chi-square, and Fisher's exact tests were used to compare percentages (including SNP frequency comparisons between cases and controls). Rho Spearman coefficients were calculated to determine correlations between analyte levels (NO, ADMA, and VEGF) and placental and neonatal weight. A *p* value of < 0.05 was considered as statistically significant.

RESULTS

During the study period a total of 62 women were recruited. These included 31 women with severe pre- eclampsia and 31 controls matched for maternal and gestational age, parity, and laboring status. General characteristics of studied women are depicted on Table I. Arm circumference (cm) as well as blood pressure levels were significantly higher among women with pre-eclampsia. No significant differences were found for the other studied parameters. None of the pre-eclampsia cases presented clinical complications (i.e. oliguria, renal insufficiency, pulmonary edema, or neurological problems). As compared to controls, at birth, placental weight and neonatal weight and ponderal index were lower in women complicated with severe pre- eclampsia (Table II). Equally the percentage of neonates with low birth weight (< 2500 g) was higher in the pre-eclampsia group.

NO plasma levels in umbilical vessels (artery and vein) were significantly higher in pre- eclampsia cases than in controls ($p = 0.0001$). There was a non-significant trend toward higher ADMA levels in both umbilical vessels among women with pre-eclampsia. VEGF levels were found to be significantly lower only in umbilical artery of pre- eclampsia cases (Table III; Figure 1a, b, and c). Artery and vein NO levels displayed a positive and significant correlation with ADMA levels. No other significant correlation was found (Table IV).

There was no statistical difference between cases and controls in the distribution of the five investigated SNPs of the VEGF gene assessed from whole blood of the umbilical venous circulation (Table V). Interestingly, lower umbilical vein VEGF levels were found in pre-eclampsia cases in the presence of the VEGF -1154 AG and -2578 CC genotypes (Table VI). No differences were observed in the distribution of any of the analyzed SNPs

or VEGF levels when pre-eclamptic women with small for gestational age fetuses were compared to those only with pre-eclampsia.

Table I. Basal characteristics of studied women.

Parameters	All (n = 62)	Cases (n = 31)	Controls (n = 31)	p Value*
Age (years)	25.7 ± 6.4 [26.0]	25.9 ± 6.4 [26.0]	25.4 ± 6.5 [26.0]	0.78 ^a
Parity	1.4 ± 1.6 [1.0]	1.4 ± 1.6 [1.0]	1.4 ± 1.6 [1.0]	1.0 ^b
Residency (%)				
Urban	11 (17.7)	6 (19.4)	5 (16.1)	0.73 ^c
Urban-marginal	35 (56.5)	19 (61.3)	16 (51.6)	0.44 ^c
Rural	16 (25.8)	6 (19.4)	10 (32.3)	0.24 ^c
Patient was in labor upon recruitment (%)	20 (32.3)	10 (32.3)	10 (32.3)	1.0 ^c
Educational level (years)	10.5 ± 3.4 [11.0]	10.8 ± 3.5 [12.0]	10.2 ± 3.4 [10.0]	0.49 ^b
Number of antenatal visits	5.4 ± 2.2 [6.0]	5.1 ± 2.0 [5.0]	5.7 ± 2.3 [6.0]	0.15 ^b
Arm circumference (cm)	28.4 ± 2.1 [28.0]	29.2 ± 2.0 [29.0]	27.6 ± 1.9 [28.0]	0.004 ^b
Systolic blood pressure (mmHg)	132.9 ± 27.3 [135.0]	156.2 ± 17.2 [150.0]	109.5 ± 9.7 [110.0]	0.0001 ^b
Diastolic blood pressure (mmHg)	83.6 ± 16.2 [85.0]	97.7 ± 7.9 [100.0]	69.5 ± 8.0 [70.0]	0.0001 ^b

Data are presented as mean ± standard deviations [medians] or percentages n (%).

*p Value after comparing cases and controls as determined with Student t-test^a, the Mann–Whitney test^b or the chi-square test^c.

Table II. Neonatal outcome of studied women.

Parameters	Cases (n = 31)	Controls (n = 31)	p Value*
Gestational age (weeks)	37.3 ± 1.9 [38.0]	37.9 ± 1.5 [38.0]	0.14 ^a
Neonatal weight (g)	2669.8 ± 623.9 [2873.0]	2945.1 ± 496.8 [2995.0]	0.02 ^b
Ponderal index (g/cm ³)	2.5 ± 0.3 [2.6]	2.8 ± 0.7 [2.8]	0.02 ^a
Low birth weight < 2500 g (%)	11 (35.5)	5 (16.2)	0.04 ^c
Preterm (%)	9 (29.0)	5 (16.2)	0.22 ^c
Small-for-gestational age (%)	5 (16.1)	1 (3.2)	0.19 ^d
Placental weight (g)	667.3 ± 57.2 [650.0]	685.6 ± 38.4 [690.0]	0.02 ^b
Apgar score < 7 at first minute (%)	7 (22.6)	2 (6.4)	0.14 ^d
Apgar score < 7 at 5 minutes (%)	2 (6.4)	0 (0)	0.49 ^e
Neonatal death (%)	0 (0)	0 (0)	0.90 ^e
Admittance to NICU (%)	0 (0)	0 (0)	0.90 ^e

Data are presented as mean ± standard deviations [medians] or percentages n (%).

*p Value after comparing cases and controls as determined with the Mann–Whitney test^a, the student t-test^b, the chi-square test^c, Yates' corrected chi-square test^d or Fisher's exact test^e. NICU, neonatal intensive care unit.

Table III. NO, ADMA, and VEGF levels in umbilical vessels: cases and controls.

Parameter	Cases	Controls	<i>p</i> Value *
Nitric oxide (mmol/L)			
Artery	4.67 ± 3.00	0.82 ± 0.90	0.0001
Vein	4.46 ± 3.00	0.82 ± 0.99	0.0001
ADMA (mmol/L)			
Artery	2.57 ± 1.03	2.34 ± 0.57	0.79
Vein	2.74 ± 0.94	2.42 ± 0.59	0.21
VEGF (pg/mL)			
Artery	200.48 ± 225.62	338.61 ± 287.03	0.04
Vein	144.49 ± 351.95	130.51 ± 235.17	0.88

**p* Value after comparing cases in controls with the Mann–Whitney test. ADMA, asymmetric dimethylarginine; VEGF, vascular endothelial growth factor.

Table IV. Rho Spearman coefficients between artery and vein analyte levels (cases and controls) and placental and neonatal weight.

	NO artery	NO vein	ADMA artery	ADMA vein	VEGF artery	VEGF vein
Cases						
ADMA	0.39 (0.04)*	0.47 (0.01)	-	-	-0.17 (0.43)	-0.07 (0.77)
VEGF	0.05 (0.79)	-0.04 (0.86)	-0.17 (0.43)	-0.07 (0.77)	-	-
Placental weight (g)	0.10 (0.63)	0.22 (0.28)	-0.26 (0.20)	-0.02 (0.90)	-0.05 (0.81)	-0.03 (0.90)
Neonatal weight (g)	-0.17 (0.40)	-0.28 (0.17)	-0.08 (0.70)	0.09 (0.65)	0.33 (0.10)	0.09 (0.65)
Controls						
ADMA	0.14 (0.48)	0.05 (0.80)	-	-	0.05 (0.80)	-0.27 (0.18)
VEGF	0.11 (0.58)	-0.32 (0.11)	0.05 (0.80)	-0.27 (0.18)	-	-
Placental weight (g)	-0.07 (0.73)	0.08 (0.67)	0.30 (0.14)	-0.14 (0.50)	0.17 (0.39)	0.04 (0.83)
Neonatal weight (g)	-0.08 (0.68)	0.04 (0.85)	-0.11 (0.60)	-0.08 (0.71)	0.32 (0.11)	-0.11 (0.59)

* *p* Values in parenthesis.

Table V. Single nucleotide polymorphisms of the promoter region of the VEGF gene among studied women.

SNP position and genotype	Pre- eclampsia		Controls		<i>p</i> Value*
	Genotype frequency	Allelic frequency	Genotype frequency	Allelic frequency	
VEGF (-2578 A/C)					
AA	3 (9.7%)	A = 35.5%	3 (9.7%)	A = 35.5%	0.66
AC	16 (51.6%)	16 (51.6%)	1.0		
CC	12 (38.7%)	C = 64.5%	12 (38.7%)	C = 64.5%	1.0
VEGF (-1498 C/T)					
CC	3 (9.7%)	C = 38.7%	4 (12.9%)	C = 38.7%	1.0
CT	18 (58.0%)	16 (51.6%)	0.60		
TT	10 (32.3%)	T = 61.3%	11 (35.5%)	T = 61.3%	0.78

SNP position and genotype	Pre- eclampsia		Controls		<i>p</i> Value*
	Genotype frequency	Allelic frequency	Genotype frequency	Allelic frequency	
VEGF (-1154 A/G)					
GG	0 (0%)	G = 12.9%	1 (3.2%)	G = 16.1%	1.0
AG	8 (25.8%)	8 (25.8%)	1.0		
AA	23 (74.2%)	A = 87.1%	22 (71.0%)	A = 83.9%	0.77
VEGF (-634 C/G)					
GG	11 (35.5%)	G = 64.5%	17 (54.8%)	G = 74.2%	0.12
CG	18 (58.0%)	12 (38.7%)	0.12		
CC	2 (6.5%)	C = 35.5%	2 (6.5%)	C = 25.8%	1.0
VEGF (+936 C/T)					
CC	15 (48.4%)	C = 67.7%	17 (54.8%)	C = 74.2%	0.61
CT	12 (38.7%)	12 (38.7%)	1.0		
TT	4 (12.9%)	T = 32.3%	2 (6.5%)	T = 25.8%	0.66

**p* Value as determined by the chi-square or the Fisher's exact test when comparing genotype frequency of cases vs. controls.

Table VI. Umbilical vein VEGF levels according to SNP genotype of the promoter region of the VEGF gene among studied women.

Position and genotype	VEGF levels (pg/mL)	VEGF levels (pg/mL)	VEGF levels (pg/mL)	<i>p</i> Value*
	all women	pre-eclampsia	controls	
VEGF (-2578 A/C)				
AA	40.9 [79.2]	52.5 [171.7]	29.2 [-]	0.70
AC	34.9 [130.3]	34.5 [151.6]	54.1 [121.6]	0.98
CC	57.6 [127.7]	33.8 [84.8]	101.1 [193.2]	0.04
<i>p</i> Value**	0.96	0.43	0.14	
VEGF (-1498 C/T)				
CC	52.5 [101.0]	52.5 [171.7]	50.5 [86.5]	0.85
CT	34.4 [139.5]	26.3 [144.6]	54.1 [145.1]	0.60
TT	66.7 [106.9]	50.9 [95.1]	93.8 [130.2]	0.19
<i>p</i> Value	0.67	0.77	0.40	
VEGF (-1154 A/G)				
GG	10.2	-	10.2 [-]	-
AG	10.6 [24.1]	8.5 [20.5]	23.5 [118.5]	0.23
AA	86.4 [128.0]	75.8 [151.0]	93.8 [123.2]	0.54
<i>p</i> Value	0.003	0.02	0.04	
VEGF (-634 C/G)				
GG	35.0 [95.8]	23.5 [193.8]	41.3 [75.1]	0.46
CG	81.1 [134.7]	50.9 [114.3]	136.7 [186.2]	0.19
CC	63.9 [186.9]	43.9 [-]	136.7 [-]	0.66
<i>p</i> Value	0.58	0.78	0.16	

Position and genotype	VEGF levels (pg/mL) all women	VEGF levels (pg/mL) pre-eclampsia	VEGF levels (pg/mL) controls	<i>p</i> Value*
VEGF (+936 C/T)				
CC	44.1 [129.8]	52.5 [165.2]	36.3 [117.1]	0.84
CT	51.3 [126.3]	16.3 [91.5]	110.4 [114.8]	0.10
TT	63.9 [146.9]	55.7 [101.8]	175.0 [-]	0.53
<i>p</i> Value	0.94	0.63	0.35	

Data are presented as medians [interquartile ranges]. **p* Value as determined by the Mann–Whitney test when comparing cases vs. controls. ***p* Value obtained using the Kruskal–Wallis test after intragroup comparison.

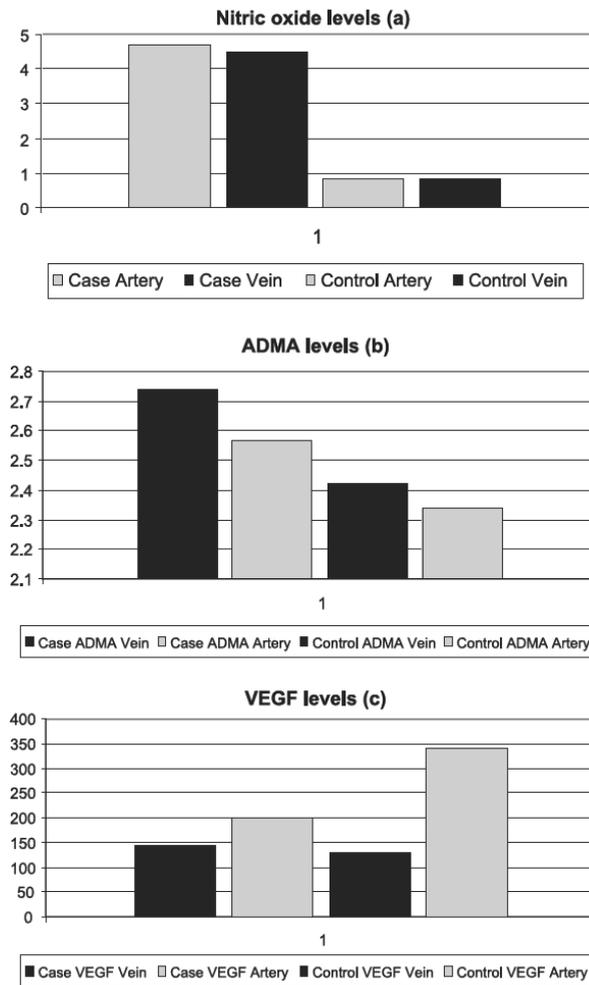


Figure 1. Levels in fetal circulation of NO (a), ADMA (b), and VEGF (c).

DISCUSSION

Endothelial dysfunction is a feature of several disorders of pregnancy. Overt maternal and feto-placental endothelial dysfunction characterizes pre-eclampsia [19], which is associated to a higher risk for adverse maternal-fetal outcomes [1,20]. Pre-eclampsia is thought to derive from shallow extravillous trophoblast invasion of the decidua and incomplete remodeling of spiral arteries and arterioles [21]. Defective angiogenesis and the related fetoplacental vascular dysfunction are therefore considered key steps. However, the signs and symptoms of pre-eclampsia generally become apparent in the third trimester of pregnancy [1], which calls for the identification of early biomarkers capable of screening women at higher risk for pre-eclampsia. Several studies have observed a correlation between the onset of vascular dysfunction and pre-eclampsia with a reduction in circulating levels of angiogenic factors, such as placental growth factor (PLGF) and VEGF [3,22], and increased levels of anti-angiogenic factors [23]. Trophoblast cells isolated from pre-eclampsia placentas when cultured under regular oxygen condition produce more soluble endoglin, soluble fms-like tyrosine kinase receptor-1, and PLGF as compared to normal trophoblast cells [23]. These changes may contribute to systemic vascular damage, possibly determining some of the modifications found in the kidney that lead to hypertension and proteinuria typical of pre-eclampsia [1].

Increased NO together with the lower VEGF umbilical plasma levels observed in the present pre-eclamptic Ecuadorian series is in agreement with what is found in the literature for other populations, however, mostly reported from maternal circulation, consistent with the general anti-angiogenic state found in pre-eclampsia [4]. To note is the fact that not many studies can be found in the literature that report fetal umbilical cord circulating levels of the analytes measured in our research. Consistent with our findings, Braekke et al. [24] have reported higher umbilical vein ADMA levels among pre-eclampsia cases as compared controls. Interestingly, ADMA levels were found to be three times higher in fetal circulation than in the maternal circulation, suggesting that the placenta is the primary source of ADMA [24]. Another study failed to demonstrate higher ADMA levels in fetal circulation in women with pre-eclampsia yet higher levels related to prematurity and low birth weight [25]. Of interest was finding in this study [25] that ADMA levels were 4 fold higher in controls as compared to those of lactating women and healthy children and adults, implicating a physiological role for higher ADMA levels in maintaining vascular tone and blood redistribution to vital organs during birth, thereby favoring the circulatory transition from fetal to neonatal life [25]. The present study cannot answer whether the higher rate of small for gestational age found in pre-eclamptic pregnancies is related to altered amounts of feto-placental growth factors. Specifically, while lower umbilical concentrations of VEGF were observed in the present and other pre-eclampsia studies, such concentrations did not significantly correlate to placental or neonatal weight.

Elevated umbilical plasma NO levels may represent an adaptive response of the fetus and placenta to maintain adequate blood supply in face of increased uterine and systemic vascular resistances and to the alleged defective angiogenesis affecting placental circulation. Whether this change is cause or consequence of pre-eclampsia is not known, but may also be seen in other pathological conditions related to pregnancy, such as fetal growth restriction, suggesting that it may rather represent a fetal response to adverse conditions imposed to pregnancy [26]. One of the biochemical changes that may elicit a feto-placental activation of the NO system may well be the increased amount of ADMA (the endogenous NOS inhibitor) which also displayed an elevated trend in umbilical blood. Indeed, previous studies have found that higher ADMA levels early during pregnancy may be associated to later development of pre-eclampsia [27].

Genetic background affects the likelihood of developing gestational hypertension and pre-eclampsia. Moreover, studies have associated pre-eclampsia to the development of subsequent cardiovascular risk [28]. Hence distribution of common SNPs of the VEGF gene was analyzed in the present research. These SNPs have also been related to cardiovascular disease [14]. Even if selected trends were noted, the limited sample size did not allow us to identify significant differences in the distribution of SNPs among analyzed groups. However, a potential strength of our study was finding a significant trend toward lower umbilical vein VEGF levels in pre-eclampsia cases in the presence of -2578 CC and -1154 AG genotypes. This is in agreement with the findings of other groups [16,29]. Contrary to this, Garza-Veloz et al. [30] have failed to demonstrate an association between pre-eclampsia and VEGF allele, genotype, or haplotype frequencies. Lower cord VEGF levels found in our *in vivo* model seem to fit well with previous *in vitro* findings reporting that in pre-eclampsia endogenous VEGF release is reduced at the placental site [31]. Although women with the 1154 allele have an increased risk for recurrent pregnancy losses [32], correlation between lower cord VEGF levels and the VEGF 1154 AG genotype is lacking in the literature.

Another interesting feature of our study was enrolling near term severe pre-eclamptic patients. About 75% of women delivering at Sotomayor Hospital have inadequate prenatal care due to low social and economic status [17]. This leads to delayed identification of pre-eclampsia and the development of overt disease, which is often diagnosed at the time of hospital admission for spontaneous labor. While most pre-eclampsia studies drawn from developed countries report data of women at lower gestational ages, our analysis shows that altered feto-placental levels of NO, ADMA, and VEGF were observed in pre-eclampsia near term.

As for the limitations of the present study one can mention the small sample size which does not allow drawing definitive conclusions in reference to the analyzed polymorphisms. Unfortunately, due to resource limitations, NOS polymorphisms were not explored, which may also be seen as a potential limitation. Nevertheless, exploring VEGF, NO, and ADMA levels and the VEGF polymorphisms on the fetal/placental side may be

seen as strengths, providing insights for future research, which should include more cases and a broader range of analyzed polymorphisms (including NOS).

In summary, this study found elevated levels of NO and ADMA in the fetoplacental circulation of pre-eclamptic women, along with lower VEGF levels. This biochemical pattern may suggest a combination of a defective angiogenesis coupled with a fetoplacental endothelial adaptive response aimed at maintaining an adequate blood flow to the fetus. A genetic predisposition toward lower umbilical vein VEGF levels is also suggested by the association with the VEGF -2578 CC and -1154 AG genotypes. These findings, despite the aforementioned limitations, contribute to the characterization of the biological basis of pre-eclampsia, and may help toward the future development of new strategies for the screening and treatment of pregnant women with this severe condition.

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Declaration of Interest

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CHAPTER 5

The role of genetics in the pathogenesis of pre- eclampsia

CHAPTER 5A

Polymorphisms of the methylenetetrahydrofolate reductase gene (C677T and A1298C) in nulliparous women complicated with pre- eclampsia.

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ABSTRACT

Objective: To determine the prevalence of C677T and A1298C Single-nucleotide polymorphisms (SNPs) of the *MTHFR* gene in nulliparous women complicated with preeclampsia (PE).

Methods: One hundred fifty gestations complicated with PE and their corresponding controls without the disease were recruited for the genotyping of C677T and A1298C polymorphisms of the *MTHFR* gene using restriction fragment length polymorphism polymerase chain reaction. Secondly, homocysteine (HCy) plasma levels were measured in preeclamptic women displaying the CC genotype of the A1298C polymorphism (homozygous) and compared to HCy levels determined among controls with the normal AA genotype for the A1298C variant.

Results: Only the mutant CC genotype of the A1298C polymorphism was associated to higher risk of presenting PE, as frequency of this genotype was significantly higher among cases than controls (15.3% versus 0.7%, $p < 0.05$). All PE women with a neck circumference ≥ 32 cm presented the mutant CC A1298C polymorphism as compared to none among preeclamptics with a lower neck circumference ($p = 0.0001$). Women with the mutant CC A1298C SNP displayed higher plasma HCy levels as compared to controls with normal AA A1298C genotype (8.4 ± 2.6 versus 7.5 ± 2.7 mmol/L $p = 0.04$).

Conclusion: Prevalence of the CC mutant genotype of the A1298C polymorphism was higher among PE women. This mutation among PE women was related to increased neck circumference and higher HCy levels. Future research should aim at linking these gestational findings with obesity and cardiovascular risk.

Keywords: Genetics, homocysteine, polymorphisms, pre-eclampsia, pregnancy

INTRODUCTION

Pre-eclampsia (PE) is a frequent complication of pregnancy that accounts for 5–14% of all deliveries, and causes adverse maternal and perinatal outcomes [1–3]. Although the exact cause of PE remains undetermined, key pathogenic factors include: immunologic factors, abnormal placental implantation, genetic and environmental factors and cardiovascular and exaggerated inflammatory changes [4–10].

Single-nucleotide polymorphisms (SNPs) are variations of the genome sequence, which may modify biological responses and the risk of certain diseases. Numerous SNPs have been studied in preeclamptic patients [5,11–15]. The methylenetetrahydrofolate reductase (MTHFR) enzyme is critical for homocysteine (HCy) metabolism, catalyzing the NADPH-linked reduction of 5,10-MTHF to 5-MTHF, and subsequently the vitamin B12-dependent methylation of HCy to methionine [5,16]. A reduction in MTHFR levels or activity by specific gene mutations induces mild to moderate increases in plasma HCy levels [17]. Although several mutations of the *MTHFR* gene have been described in relation to PE, the most frequent ones include: the alanine-to-valine C677T [18] and the glutamate-to-alanine A1298C [19]. While both SNPs induce milder forms of MTHFR deficiency [19,20], the A1298C SNP (located in the enzyme regulatory domain) as compared to the C677T variant (located in the enzyme catalytic domain), does not result in either a thermolabile protein or increased HCy blood levels [21]. Interestingly, reports indicate that C677T/A1298C compound heterozygosity has similar clinical impact as C677T homozygosity [20,22].

Data related to the genetic assessment of women complicated with PE from developing countries are scarce or lacking. Hence, the aim of this study was to determine the prevalence of C677T and A1298C polymorphisms of the MTHFR gene in nulliparous women complicated with PE. Secondly, plasma HCy levels were measured in preeclamptic women displaying the homozygous CC genotype of the A1298C polymorphism and compared to HCy levels determined among controls with the normal AA genotype for the A1298C variant.

METHODS

Design and subjects

This research was carried out at the Institute of Biomedicine, Universidad Católica de Santiago de Guayaquil in collaboration with the Enrique C. Sotomayor Obstetrics and Gynecology Hospital, Guayaquil, Ecuador. This Hospital is a major referral center that provides maternal and neonatal healthcare to the low-income population of Guayaquil, Ecuador. This facility has one of the highest delivery rate in Latin America (> 30 000 per year) in addition to increased rates of high-risk pregnancies.

Nulliparous women with singleton gestations fulfilling PE criteria admitted for delivery to the High Risk Pregnancy Labor and Delivery Unit were recruited. Nulliparous women without PE delivering in the low risk Unit served as controls. All participants were recruited after signing informed consent. Women denying participating were excluded from the study.

ACOG criteria was used to define PE as a blood pressure $\geq 140/90$ mmHg and proteinuria + or more on at least two random samples four hours apart. PE was defined as severe if blood pressure was $\geq 160/110$ mmHg and proteinuria was +++ or more [3]. Maternal socio-demographic, pregnancy and obstetrical and neonatal outcome data was recorded on a specific data sheet. Research study protocol was reviewed and approved by the Institutional Review Board of the Enrique C. Sotomayor Hospital.

Blood sampling and biochemical assay

A 10–15 ml peripheral venous blood sample was obtained from each participant, which was then centrifuged at 5° C for 10 min at 3000 rpm. Obtained plasma and white cells were decanted into 0.5 ml aliquots, which were then stored at -70° C until further analysis. Hcy plasma levels were measured with a microparticle chemiluminescent immunoassay using an ARCHITEC autoanalyzer (ARCHITEC *i* System, Abbott Diagnostic Laboratories, Abbott Park, Chicago, IL).

Single nucleotide polymorphism (SNP) genotyping

DNA was extracted from whole blood using the PureLink genomic kit (Invitrogen®, Carlsbad, CA), which was then amplified through conventional polymerase chain reaction (PCR) technique using the following primers: 5'TGAAGGAGAAGGTGTCTGCGGA3' and 5'AGGACGGT GCGGTGAGAGTG3' for C677T; and 5'TGATGAAATCGGC TCCCGCA3' and 5'TGATGATGAAATCGACTCCCGCA3' for A1298C. The amplified product (a 198 bp fragment of exon 4 of the *MTHFR* gene) was used for the genotyping of the C677T and A1298C polymorphisms using restriction fragment length polymorphism PCR. Allelic distribution for C677T and A1298C SNPs was in Hardy–Weinberg equilibrium.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences version 19.0 (IBM SPSS, Armonk, NY). Data are presented as mean \pm standard deviations, frequencies, percentages, odds ratios and 95% confidence intervals. The Kolmogorov–Smirnov test was used to determine the normality of data distribution. According to this, continuous data were compared with student's *t* test (parametric) or the Mann Whitney *U* test (non parametric). Chi-square, Yates' corrected chi-square and Fisher's exact tests were used to compare percentages (including the comparison of genotype

frequencies between cases and controls). A p value of <0.05 was considered as statistically significant.

RESULTS

A total of three hundred pregnant women were recruited during the study period ($n = 150$ cases of PE and $n = 150$ controls). Socio-demographic and obstetrical outcome data of studied women are depicted on Table 1. Women with PE lived more frequently in rural areas or had a family history of hypertension and had fewer prenatal visits than controls. Anthropometric measures (neck and mid-arm circumference) were significantly higher in PE women. C-section rate was also significantly increased among women with PE as compared to controls.

Neonatal gestational age and weight was significantly lower among PE women. The rate of preterm birth, small for gestational age infants, low birth weight and low Apgar scores (first and five minutes) were significantly higher among PE women as compared to controls (Table 2). There were no neonatal deaths in either studied groups.

Genotype frequencies of the studied *MTHFR* gene polymorphisms (C677T and A1298C) among cases and controls are depicted on Table 3. No significant differences were observed among cases and controls in relation to genotype frequencies for the C677T polymorphism. Contrary to this, the mutant CC genotype of the A1298C polymorphism was associated to a higher risk of presenting PE (OR: 25.3, 95% CI: 3.53–512.4, $p = 0.0001$), as prevalence of this genotype was found to be significantly higher among PE cases as compared to controls (15.3% versus 0.7%, $p < 0.05$). Among cases, frequency of C677T and A1298C genotypes did not differ when women were stratified according to PE severity, the presence of a small for gestational age neonate or history of family hypertension. However, all PE women with a neck circumference ≥ 32 cm presented the mutant CC genotype of the A1298C polymorphism as compared to none among PE women with a neck circumference < 32 cm ($p = 0.0001$). Women with the mutant CC genotype A1298C polymorphism displayed higher plasma Hcy levels as compared to controls with the normal A1298C genotype (homozygous for AA) (8.4 ± 2.6 versus 7.5 ± 2.7 mmol/L $p = 0.04$, Mann–Whitney U test).

Table 1. Socio-demographic and obstetrical outcome data of studied women.

Socio-demographic	Cases n = 150	Controls n = 150	<i>p</i> Value*
Age (years)	19.9 ± 5.0	19.4 ± 4.4	0.33
Cohabiting	97 (64.7)	90 (60.0)	0.40
Lives in rural area	70 (46.7)	44 (29.3)	0.001
Educational level (years)	10.1 ± 3.0	10.0 ± 3.2	0.76
Alcohol consumption during pregnancy	5 (3.3)	7 (4.7)	0.55
Cigarette consumption during pregnancy	1 (0.7)	3 (2.0)	0.61
Employed	16 (10.7)	16 (10.7)	1.0
Age at menarche (years)	12.7 ± 1.3	12.6 ± 1.4	0.62
Sometime contraceptive use	23 (15.3)	20 (13.3)	0.62
Family history of hypertension	37 (24.7)	14 (9.3)	0.0001
Number of prenatal visits	5.1 ± 2.3	5.9 ± 2.1	0.002
Pregnancy and obstetrical outcome			
Systolic blood pressure (mmHg)	151.8 ± 17.6	109 ± 12.3	0.0001
Diastolic blood pressure (mmHg)	99.1 ± 15.0	68.1 ± 10.0	0.0001
Severe pre- eclampsia	110 (73.3)	–	
Mild pre- eclampsia	40 (26.7)	–	
Neck circumference (cm)	33.6 ± 3.5	31.5 ± 3.1	0.0001
Neck circumference ≥ 32 cm (median)	114 (76.0)	76 (50.7)	0.0001
Mid-arm circumference (cm)	27.8 ± 4.0	25.9 ± 3.3	0.0001
Delivery by c-section	121 (80.7)	53 (35.3)	0.0001

Data are presented as mean ± standard deviations or *n* (%).

**p* Value as determined according to case with the Mann–Whitney *U* test, the Student's *T* test or the chi-square or Yates' corrected chi-square test.

Table 2. Neonatal outcome among studied women.

Parameter	Cases n = 150	Controls n = 150	<i>p</i> Value*
Gestational age (weeks)	36.7 ± 3.3	38.7 ± 1.7	0.0001
Neonatal birthweight (gr)	2406.1 ± 765.5	2975.3 ± 502.4	0.0001
Female gender	77 (51.3)	74 (49.3)	0.72
Stillbirths	4 (2.7)	0 (0.0)	0.12
Preterm	47 (31.3)	12 (8.0)	0.0001
Small for gestational age	61 (40.7)	15 (10.0)	0.0001
Low birthweight (< 2,500 gr)	72 (48.0)	26 (17.3)	0.0001
Apgar <7 at first minute	48 (32.0)	15 (10.0)	0.0001
Apgar <7 in five minutes	21 (14.0)	3 (2.0)	0.0001
Admittance to NICU	8 (5.3)	3 (2.0)	0.12

Data are presented as mean ± standard deviations or *n* (%).

**p* Value as determined according to case with the Mann *U* Whitney test, Student's *T* test, chi-square or Fisher's exact test. NICU, neonatal intensive care unit.

Table 3. Genotype frequencies of the studied *MTHFR* gene polymorphisms (C677T and A1298C) among cases and controls.

Genotype	Cases <i>n</i> = 150	Controls <i>n</i> = 150	Odds ratio	95% CI	<i>p</i> Value
C677T					
CC	59 (39.3)	47 (31.3)	1		
CT	73 (48.7)	91 (60.7)	0.64	0.38–1.08	0.07
TT	18 (12.0)	12 (8.0)	1.19	0.49–2.95	0.67
CT + TT	91 (60.7)	103 (68.7)	0.70	0.43–1.16	0.14
A1298C					
AA	100 (66.7)	110 (73.3)	1		
AC	27 (18.0)	39 (26.0)	0.76	0.42–1.38	0.34
CC	23 (15.3)	1 (0.7)	25.30	3.53–512.4	0.0001
AC + CC	50 (33.3)	40 (26.7)	1.38	0.81–2.33	0.2

Data are presented as *n* (%) and odds ratios and 95% confidence intervals (CI).

DISCUSSION

PE is a mysterious disease that produces severe or even fatal obstetrical complications and beyond pregnancy it has been associated to future increased cardiovascular risk [23–26]. In developing countries, such as Ecuador, its prevalence tends to be higher than in developed populations and in relation to factors such as inadequate prenatal care, genetic factors, ethnicity, malnutrition and low socio-economical income [3,27–29]. Highlighting this, our PE cases displayed fewer prenatal visits and higher rates of rural residency and family history of hypertension and adverse maternal and neonatal outcomes.

This study aimed at examining among nulliparous women complicated with PE the prevalence of two known SNPs of the *MTHFR* gene (C677T and A1298C). The prevalence of the mutant CC genotype of the A1298C polymorphism was found to be higher in our PE women as compared to controls. Prevalence did not vary in accordance to disease severity nor to the presence of small gestational age neonates or a family history of hypertension. In addition, women with this mutant genotype displayed higher plasma Hcy levels as compared to controls with the normal AA genotype (A1298C). These findings correlate with those observed in a group of Tunisian women with several pregnancy complications including recurrent pregnancy loss, PE, placental abruption and fetal intrauterine growth restriction [17].

The *MTHFR* enzyme catalyzes the irreversible conversion of 5,10-MTHF into 5-MTHF, which is the methyl donor for remethylation of Hcy to methionine. Certain common polymorphisms within the *MTHFR* gene (C677T or A1298C) result in reduced enzymatic remethylation of Hcy to methionine and, consequently, are associated with higher Hcy blood levels [24]. Around 30% of Europeans, 10% of Africans and 50% of

Chinese people display C677T and/or A1298C variants of the *MTHFR* gene, with increased cardiovascular risk and altered metabolic processes among homozygous cases [18–22,30]. Although reports indicate that the A1298C variant seems to only cause problems when it is associated with the C677T variant [31], our study found no difference in the prevalence of the C677T genotypes when PE women were compared to controls. Other *MTHFR* variants have been less studied and are probably not of relevance.

In a meta-analysis including 51 studies, the C677T *MTHFR* polymorphism was associated to an overall increased risk for PE among Caucasian and East Asian populations. Unfortunately, the study only analyzed a small sample for Latin, East Asian, South Asian and African populations [32]. In a small sample of a Mexican Maya-Mestizo population, the *MTHFR* 677T allele and the 677TT genotype were significantly more frequent among controls, suggesting an association with a decreased risk of PE [33]. This study is to the best of our knowledge the first to assess *MTHFR* polymorphisms among PE complicated gestations within a low-income Mestizo Ecuadorian population in which although prevalence of the A1298C mutant CC genotype was found to be higher among PE cases, the mutant TT genotype for C677T was not. Our results are in agreement with others showing that C677T polymorphisms did not correlate to uterine artery Doppler values and bilateral or unilateral notch occurrences at 12 and 22 weeks of pregnancy [34], or the risk of intrauterine fetal death, PE and preterm delivery [35–38].

Our study not only found that the CC mutant genotype of the A1298C variant was significantly associated with PE yet these women displayed higher Hcy plasma levels. In accordance to this, Klai et al. [17] have reported that women with the *MTHFR* A1298C mutation have a higher risk of placental vasculopathies and elevated Hcy levels. Contrary to this, Said et al. [39] reported among an Australian cohort that nulliparous women with homozygous *MTHFR* A1298C polymorphism had a lower risk for poor obstetric outcomes (PE, fetal growth restriction, placental abruption and stillbirth).

Hyperhomocysteinemia and folic acid and vitamin B₁₂ deficiency along with increased blood pressure are risk factors for thrombotic events and the development of PE [40,41]. The mechanisms underlying hyperhomocysteinemia may relate to genetic polymorphisms, vitamin B₁₂ and folic acid deficiency, oxidative stress and endothelial dysfunction. We have previously reported that PE patients display significantly lower erythrocyte folic acid levels together with higher serum Hcy levels as compared to controls [42]. Hyperhomocysteinemia and *MTHFR* polymorphisms in women with PE have previously been reported, fact that is in agreement with our data. Despite the aforementioned, hyperhomocysteinemia itself cannot be solely explained by those mutations [43–46]. It is clear that women with PE have higher Hcy blood concentrations than those with uncomplicated pregnancies; however, as found in our series, relationship between Hcy levels and PE severity is not clear [47].

The global risk of PE increases significantly with increasing BMI and younger age. Thus, extreme obese teenagers have almost four times the risk of developing PE as

compared to nonobese pregnant women aged 20–24 years [46]. Obesity is also a risk factor for preterm birth; and in multivariable regression analysis, the most important intermediate variable is PE [48]. In this study, two indirect measures of obesity – neck and mid-arm circumference – were significantly higher in cases than in the controls suggesting an association between obesity and PE risk. Ursavas et al. [49] have reported neck circumference as an independent risk factor for gestational hypertension and PE. In addition, our series found that a higher neck circumference, thus increased weight (cut off value of 32 cm), was associated with a higher prevalence of the mutant CC genotype A1298C variant and moreover these women displayed higher HCy levels. Elucidating a link between these gestational findings and future cardiovascular risk is certainly wanted.

As for the limitations of this study, one can mention the lack of vitamin B12 and folic acid measurements, which would have given more information about the individual metabolic status and its relation to the studied polymorphisms. Analyzing pro-inflammatory cytokines and adipocytokines (i.e. leptin) would have aided at giving more insights regarding the relationship between PE, obesity and cardiovascular risk [46,50–52]. Finally, another concern is the difficulty to assessing obesity in pregnant women [53], moreover if one lacks prenatal or early pregnancy weight. Despite this, we obviated this problem by using indirect anthropometric parameters of obesity, which have been validated by others [54,55]. Despite these limitations, our study has the strength of being the first to provide data of the genetic assessment of polymorphisms in PE-complicated gestations from a homogeneous low-income Ecuadorian Mestizo population.

In conclusion, prevalence of the CC mutant genotype for the A1298C polymorphism was higher among PE women. This mutation among PE women was related to increased neck circumference and higher HCy levels. Future research should aim at linking these gestational findings with obesity and cardiovascular risk.

Authors' role

P. C., F. R. P-L. and T. S.: study conception and design; D. S-P and A. V acquisition of clinical data; G. S. E and C. R. laboratory work; P. C., L. H., F. R. P-L., A. G. and T. S.: analysis and interpretation of data; P. C. and F. R. P-L.: drafting of manuscript; L. H. and A. G.: critical revision; P. C. and F. R. P-L.: final adjustments; all authors approved the final version.

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Declaration of interest

The authors declare no conflicts of interest.

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CHAPTER 5B

Polymorphisms of the methylenetetrahydrofolate reductase gene (C677T and A1298C) in the placenta of pregnancies complicated with pre- eclampsia.

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ABSTRACT

Background: Pre- eclampsia has been related to single-nucleotide polymorphisms (SNPs) of the methylenetetrahydrofolate reductase (MTHFR) gene; however, data regarding the placenta are still lacking.

Objective: To determine the frequency of C677T and A1298C SNPs of the MTHFR gene in the placenta of preedamptic pregnancies and healthy controls.

Methods: Genotyping of C677T and A1298C polymorphisms of the MTHFR gene using RFLP-PCR was performed to the placenta of 100 gestations ($n = 50$ complicated with pre- eclampsia and $n = 50$ normal controls matched for parity and maternal age).

Results: Gestational age at birth and neonatal and placental weight were significantly lower in women with pre- eclampsia as compared to controls. The TT genotype of the C677T polymorphism was threefold more prevalent in preeclamptic placentas as compared to the placenta of controls (24.0% versus 8.0%, $p = 0.001$). Upon pooled analysis ($n = 100$), placental and neonatal weights were significantly lower in placentas displaying this genotype (TT, C677T) as compared with the CC genotype.

Conclusion: This study found that the frequency of the TT mutant genotype of the C677T polymorphism was higher in the placenta of pregnancies complicated with pre-eclampsia. There is a need for further research in this matter.

Keywords: Genetics, homocysteine, pre- eclampsia, placenta, polymorphisms, pregnancy

INTRODUCTION

Pre-eclampsia (PE) is a frequent complication of pregnancy related to adverse maternal-fetal outcomes [1]. To date, the exact cause of PE remains undetermined; however, various key factors or abnormalities have been elucidated: genetic or environmental factors, inflammatory and immunologic factors, phenotypical cardiovascular mechanical risk factors and biochemical cardiometabolic risk factors amongst hyperhomocysteinemia [2-4].

Several single-nucleotide polymorphisms (SNPs) have been studied in pregnancies complicated with PE [5-7]. The methylenetetrahydrofolate reductase (MTHFR) enzyme is critical for the metabolism of homocysteine (HCY), catalyzing the NADPH-linked reduction of 5,10-MTHF to 5-MTHF, and subsequently the vitamin B12-dependent methylation of HCY to methionine [8]. Lower levels or activity of this MTHFR enzyme, due to specific gene mutations, induces mild to moderate increases in plasma HCY levels [9]. Two specific SNPs of the MTHFR gene have been reported: C677T (leading to valine substitution at amino acid 222) [10], and A1298C (leading to an alanine substitution at amino acid 429) [11]. Both mutations have been studied in women with PE [12,13]. We previously reported that the prevalence of the CC mutant genotype of the A1298C polymorphism, yet not the TT mutant genotype of C677T, was higher among women with PE [12]; and this mutation was related to higher maternal plasma HCY levels. Not many studies have reported on these mutations in the placental tissue of gestations complicated with PE. Therefore, to complement our preliminary maternal findings [12], the present research aimed at determining the frequency of the C677T and A1298C SNPs of the MTHFR gene in the placenta of pregnancies complicated with PE and healthy pregnant controls.

METHODS

Study design and participants

The present research was carried out at the Institute of Biomedicine, Universidad Católica de Santiago de Guayaquil, Ecuador, with the support of the School for Oncology and Developmental Biology Maastricht (GROW), Maastricht University, the Netherlands. Women were recruited from the Enrique C. Sotomayor Obstetrics and Gynecology Hospital (Guayaquil, Ecuador), which is a major referral center providing maternal and neonatal healthcare to the low-income population. This facility has an important number of deliveries and provides care to many high-risk pregnancies.

Women with singleton gestations fulfilling PE criteria admitted for delivery to the High Risk Pregnancy Labor and Delivery Unit were invited to participate. Those without PE delivering in the Low Risk Labor Unit served as controls matched for parity and ma-

ternal age. All subjects were informed about the study and its aims, providing signed consent of participation.

American Congress of Obstetricians and Gynecologists (ACOG) criteria were used to define PE as a blood pressure $\geq 140/90$ mmHg and proteinuria + or more on at least two random samples 4 h apart [14]. PE was defined as severe if blood pressure was $\geq 160/110$ mmHg and proteinuria was +++ or more [14]. Maternal socio-demographic as well as obstetrical and neonatal data were recorded on a specific data sheet. The research protocol was reviewed and approved by the Institutional Review Board (IRB) of the Enrique C. Sotomayor Hospital.

Placental sampling and SNP genotyping

After delivery, the placenta was weighed and a 25 g sample was obtained and immediately stored at -70°C until analysis. Subsequently, DNA was extracted from the placental sample using the PureLink genomic kit (Invitrogen®, Carlsbad, CA), which was then amplified through conventional polymerase chain reaction (PCR) technique using the following primers: 5'-TGAA GGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGG TGAGAGTG-3' for C677T; and 5'-TGATGAAATCGGCTC CCGCA-3' and 5'-TGATGATG AAATCGACTCCCGCA-3' for A1298C. The amplified product (a 198 bp fragment of exon 4 of the MTHFR gene) was used for the genotyping of the C677T and A1298C polymorphisms using restriction fragment length polymorphism PCR (RFLP-PCR). Allelic distribution for C677T and A1298C SNPs was in the range of Hardy-Weinberg equilibrium.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences version 22.0 (IBM SPSS, Armonk, NY). Data are presented as medians, interquartile ranges [IRQ], frequencies, percentages, odds ratios and 95% confidence intervals. The Kolmogorov-Smirnov test was used to determine the normality of data distribution. According to this, non parametric continuous data were compared with the Mann-Whitney *U*-test. Chi-square, Yates' corrected chi-square or Fisher's exact tests were used to compare percentages (including the comparison of genotype frequencies between cases and controls). A *p* value of < 0.05 was considered as statistically significant.

RESULTS

A total of 100 pregnant women were recruited during the study period ($n = 50$ cases of PE and $n = 50$ controls). Socio-demographic and obstetrical/neonatal data of the studied women are depicted in Table 1. C-section rate was higher among women with PE as compared to controls. Women with PE had more often adverse perinatal outcomes.

Gestational age at birth and neonatal and placental weight were lower in PE cases. In addition, rates of preterm, low birth weight and low Apgar scores were significantly higher in PE women.

Table I. Socio-demographic and obstetrical/neonatal data of the studied women.

Socio-demographic	Cases <i>n</i> = 50	Controls <i>n</i> = 50	<i>p</i> Value*
Age (years)	25.5 [13]	26.0 [10.5]	0.91
Parity	1.0 [4]	2.0 [2.5]	0.74
Number of prenatal visits	4.0 [5]	5.0 [4.5]	0.62
Obstetrical data			
Systolic blood pressure (mmHg)	149.5 [20.5]	100.0 [11.0]	0.0001
Diastolic blood pressure (mmHg)	100.0 [10.5]	65.0 [11.0]	0.0001
Severe pre- eclampsia	43 (86.0)		
Mild pre- eclampsia	7 (14.0)		
Delivery by c-section	45 (90.0)	24 (48.0)	<0.001
Placental weight (g)	520.0 [235.5]	626.5 [129.5]	0.001
Neonatal data			
Gestational age (weeks)	37.5 [5.0]	39.0 [1.5]	0.0001
Neonatal birthweight (g)	2,538 [1,313.5]	3,044 [638.0]	0.0001
Female gender	24 (48.0)	19 (38.0)	0.41
Preterm birth	19 (38.0)	2 (1.0)	<0.0001
Low birth weight (< 2,500 g)	23 (46.0)	5 (10.3)	0.0001
Apgar < 7 at first minute	12 (24.0)	1 (2.0)	0.001
Apgar < 7 in five minutes	7 (14.0)	0 (0.0)	0.01

Data are presented as medians [interquartile ranges] or frequencies (%); **p* Value as determined depending on the case with the Mann-Whitney *U* test or the chi-square, Yates' corrected chi-square or Fisher's exact test.

Genotype frequencies of the studied MTHFR gene polymorphisms (C677T and A1298C) in the placenta of cases and controls are depicted in Table 2. No significant differences were observed among cases and controls in relation to genotype frequencies for the A1298C polymorphism. Contrary to this, the mutant TT genotype of the C677T polymorphism was threefold more prevalent in preeclamptic placentas as compared to the placenta of controls (24.0% versus 8.0%, *p* = 0.001). Pooled analysis (*n* = 100) showed that placental and neonatal weights were significantly lower in placentas displaying the TT genotype of the C677T polymorphism as compared with the CC genotype (Data not shown in the table).

Table 2. Genotype frequencies of the studied MTHFR gene polymorphisms (C677T and A1298C) in the placenta of cases and controls.

Genotype	Cases <i>n</i> = 50	Controls <i>n</i> = 50	Odds ratio	95% CI	<i>p</i> Value
C677T					
CC	15 (30.0)	20 (40.0)	1		
CT	23 (46.0)	26 (52.0)	1.18	0.45-3.10	0.71
TT	12 (24.0)	4 (8.0)	4.0	1.09-14.89	0.001
CT+TT	35 (70.0)	30 (60.0)	1.56	0.63-3.87	0.29
A1298C					
AA	37 (74.0)	39 (78.0)	1		
AC	11 (22.0)	8 (16.0)	1.45	0.47-4.50	0.47
CC	2 (4.0)	3 (6.0)	0.70	0.08-5.60	0.70
AC+CC	13 (26.0)	11 (22.0)	1.25	0.45-3.45	0.63

Data are presented as frequencies (%), odds ratios and 95% confidence intervals (CI).

DISCUSSION

PE is one of the major complications of pregnancy and the leading cause of maternal and fetal morbidity and mortality [1]. Although the cause of PE remains unknown, studies have highlighted the fact that a failure in placentation very early in gestation leads to the release of several products and factors that cause the syndrome through a multiplicity of underlying pathophysiological mechanisms [15,16]. Despite this, the importance of genetics in the origin of the disease is currently gaining interest. Indeed, the most plausible genetic model postulates that the occurrence of the pathological phenotype in PE depends on the maternal genetic susceptibility, trophoblastic genetic load and various environmental factors [17].

Recent studies indicate that PE increases maternal and fetal future cardiovascular risk [18]. On the one hand, both traditional cardiovascular and cardiometabolic risk factors consistent with the metabolic syndrome have been linked to vascular derangements, pre-eclampsia and cardiovascular disease; and on the other hand, higher HCY levels been linked to PE development [19] and also adult cardiovascular risk [20]. Polymorphisms of the MTHFR gene (i.e. C677T and A1298C) cause lower enzyme activity resulting in a reduced remethylation of HCY to methionine, and consequently higher HCY blood levels [21]. We previously reported a higher prevalence of the CC mutant genotype of the A1298C polymorphism in whole blood of women with PE. These women displayed higher plasma HCY levels. Contrary to this, the present study found a higher frequency of the TT mutated genotype of the C677T polymorphism in the placental tissue of those complicated with PE. There are no sufficient data related to genotypes of these MTHFR polymorphisms in placental material and the role that placental abnor-

malities of folate metabolism could have in the pathogenesis of PE. Nevertheless, placental genotype and phenotype define the final set of metabolites reaching the fetus and from the fetus to the maternal circulation. In this sense, Mislanova et al. [22] determined a non-significant trend for a higher prevalence of the TT genotype of the C677T polymorphism in the placental samples of PE women as compared with controls. In essence, their finding is in correlation with ours; however, we did not quantify HCY in the placental samples. Their study [22] found a higher placental HCY content among PE complicated pregnancies expressing the placental CT genotype of the C677T polymorphism. Interestingly our study found that independent of presenting PE or not (pooled analysis $n = 100$), placental and neonatal weights were significantly lower in placentas displaying the mutant TT genotype of the C677T polymorphism as compared with the CC normal genotype. Pre- eclampsia and fetal growth restriction are both considered placental syndromes [23], in which the maternal syndrome relates to excess of placental shedding whereas the fetal syndrome related to placental dysfunction. In this sense, the TT polymorphism seems to relate to both the maternal and fetal placental syndromes. On the one hand, if we assume a defective first trimester placental invasion to underlie the later maternal and fetal clinical sequelae, then apparently the TT genotype relates to faulty placental growth. On the other hand, if exaggerated placental damage leads to loss in function and increased detaching of placental debris, then also this TT genotype gives rise to extrinsic damage or intrinsic increased sensibility to stressors. In any case, the TT genotype seems to increase an overall risk of the placental syndrome. If we hypothesize that the related placental dysfunction is due to higher HCY production in those expressing the TT mutant type, then one should expect a positive effect of folic acid, pyridoxine and vitamin B12 over phenotypical placental modulation in the reduction of noxious HCY increased levels; nevertheless, this remains to be elucidated. Despite our findings, the causative role of MTHFR gene polymorphisms (in the mother or the placenta) for the development of PE is still a subject under debate and requires further research.

Finally, as for the limitations of the present study, one can mention the lack of determining the content of folate and that of other related metabolites in placenta that would have allowed determining functional phenotypical differences affecting both fetus and mother according to the genotype characterization. Despite these limitations, the present research has two strengths: (a) our data adds to the few assessing MTHFR gene polymorphisms in placental material of women complicated with PE and (b) it is the first to provide this type of analysis among Latin American pregnant women. Certainly, our findings require further research.

In conclusion, this study found that the frequency of the TT mutant genotype of the C677T polymorphism was higher in the placenta of pregnancies complicated with PE. There is a need for further research in this regard.

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Declaration of interest

The authors declare no conflicts of interest.

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CHAPTER 6

Discussion and summary

PE is a serious problem occurring during women's reproductive years imposing a negative impact on maternal and fetal outcome [1]. Despite intensive research involved pathogenic mechanisms are still unknown and there is no treatment other than pregnancy termination. PE also exerts a negative impact on the healthcare system of both developed and non developed countries. Moreover, the poverty conditions observed in Latina American impose a greater challenge as the negative impact, in the mother, the fetus and the healthcare system seems to be greater. The present research aimed at assessing various biochemical and molecular markers related to vascular, anti-oxidative, endothelial, inflammatory and genetic abnormalities found in gestations complicated with PE.

Vascular homeostasis and inflammation during third trimester of preeclamptic women were analyzed in the study presented in Chapter 2, in which maternal plasma levels of two soluble anti-angiogenic factors (sFlt-1 and sEng) and four pro-inflammatory cytokines (IL-6, IL-8, G-CSF and TNF- α) were measured in near term nulliparous women complicated with PE. These levels were compared to those found in matched normal nulliparous gestations. It was found that increased sFlt-1 and sEng levels in maternal plasma are consistent with vascular dysfunction found in gestations complicated with PE. Elevated anti-angiogenic levels of sFlt-1 and sEng seem to contribute to maternal vascular dysfunction by binding to and thus reducing circulating levels of angiogenic factors such as free VEGF and PLGF [2]. The resulting anti-angiogenic milieu is implicated in inducing systemic vascular damage, particularly as a target the kidney to induce hypertension and proteinuria of PE [1]. Contrary to expected, PE women displayed lowered IL-8 and G-CSF levels. Our data, regarding sFlt-1 and sEng are in correlation with those found by others in relation to third trimester complicated PE women [2-4]. To note is the fact that currently sFlt is being combined with other biochemical and biophysical markers for first trimester screening of PE [5].

During PE there is an observed increase in the rate of lipid peroxidation, increased lipid availability, and a decrease of several antioxidants such as alpha tocopherol, ascorbate, beta carotene and selenium [6]. In this sense, CoQ₁₀ is a part of the non-enzymatic defense system against oxygen species (antioxidative function) [7], playing a key role in mitochondrial complexes I and III reaction mechanisms (electron transport) [8]. Two previous investigations have found lower CoQ₁₀ plasma levels [9] and lower CoQ₁₀ plasma together with higher placental CoQ₁₀ content in women complicated with PE living at high altitude [10]. As a continuation, CoQ₁₀ levels were measured both in plasma and placenta of normal pregnant and PE primigravid women residing either at high or low altitude (Chapter 3). Data analyzing CoQ₁₀ content in plasma and placenta with altitude as a component in the equation is scarce in the literature. It was found that women with PE (high or low altitude) displayed high placental CoQ₁₀ content, with significant lower plasma CoQ₁₀ levels among those residing in high altitude. Correlating with our data, higher placental CoQ₁₀ content was found among sea level residing Italian pregnant women with HELLP syndrome (a severe complication of pre-eclampsia) when

compared to normal pregnancies; however, plasma levels were not measured [11]. Our data allows us to hypothesize that CoQ₁₀ plasma levels are not directly related to placental CoQ₁₀ content. Moreover, it is also plausible to assume that placental development is totally an energy dependent process and that CoQ₁₀ could be an essential element in its physiological role. If this is true, then the next obvious questions to be considered should be: what occurs during PE? Are these high placental levels a late compensatory mechanism?

Placental dysfunction, due to defective spiral artery invasion, already present in the first trimester of women destined to develop PE, leads to a progressive maternal state of inflammation and endothelial dysfunction, which eventually predicts the severity of maternal fetal outcome [12]. Several factors may be important in the physiological mechanisms of placental angiogenesis and regulation of vascular tone, and these factors could have major roles in the pathogenesis of abnormal placental functioning. One of these factors is NO, the intracellular gaseous messenger synthesized by NOS from L-arginine and oxygen. NO exerts diverse biological functions in several physiological and pathological processes, especially in vascular pathophysiology [13]. NO produced by syncytiotrophoblast-derived endothelial cells is thought to cause dilation of the human placental vasculature [14], and may act as a paracrine agent for the maintenance of uterine quiescence during pregnancy. Additionally, local placental NO generation may be essential to promote cytotrophoblast endovascular invasion, an essential feature of normal placentation [15]. Chapter 4 aimed at evidencing by means of two study the state of maternal and fetal endothelial dysfunction. In first study NO content in plasma and placenta was measured in the third trimester of PE complicated women as compared to normal control; however, again as a highlighted feature, the effect of high or low altitude was analyzed. Independent of the site of residency women with PE presented higher NO content in plasma and placenta, which is consistent with our previous findings [16]. When women were analyzed by site of residency a similar trend for higher plasma and placental NO levels were observed in PE cases. Nevertheless, higher plasma NO levels were found in those living at sea level and higher placental NO levels observed in those living at high altitude. It is important to mention the fact that studies measuring placental NO levels, either at sea level or at altitude, are lacking. Thus, it can be hypothesized that NO levels differ not only in the presence of PE, but are also dependent on the women's altitude of residence. It is worthy to mention that contradictory results are reported in the literature regarding NO and PE, some indicating higher [16] and others lower maternal levels [17]. Although this may be due to measurement methods and/or analyzed metabolite, agreement exists that PE is related to an altered NO production (dysfunctional endothelia) and/or activity (compensatory endothelial mechanism).

In a subsequent study we further measured markers of endothelial function (NO, ADMA and VEGF) in the fetal circulation of women complicated with severe PE. In addition, genetic assessment was performed and DNA extracted from the umbilical vein to

determine the frequency of VEGF gene single nucleotide polymorphisms. Important to mention is that molecular analysis of endothelial function has only been performed in women with PE in the maternal side [18]; hence, umbilical fetal circulation data are scarce or lacking. Our study found that women with severe PE displayed higher NO and ADMA fetal circulating levels (vein and artery) and lower VEGF umbilical vein levels, which is consistent with our maternal observations. Overall, the frequency of the studied VEGF gene polymorphisms did not differ among PE cases and controls; nevertheless, a significant trend toward lower umbilical vein VEGF levels was observed in PE cases in the presence of -2578 CC and -1154 AG genotypes. Elevated umbilical plasma NO levels may represent an adaptive response of the fetus and placenta to maintain adequate blood supply in face of increased uterine and systemic vascular resistances and to the alleged defective angiogenesis affecting placental circulation. Whether this change is cause or consequence of PE is not known, but may also be seen in other pathological conditions related to pregnancy, such as fetal growth restriction, suggesting that it may rather represent a fetal response to adverse conditions imposed to pregnancy [19].

There is increasing evidence that highlight the important role of genetics and epigenetics in the development and severity of the outcome of PE. SNPs are variations of the genome sequence, which may modify biological responses and the risk of certain diseases. Numerous SNPs have been studied in women with PE [20-22]. The MTHFR enzyme is critical for Hcy metabolism, catalyzing the NADPH-linked reduction of 5,10-MTHF to 5-MTHF, and subsequently the vitamin B12-dependent methylation of Hcy to methionine [20]. A reduction in MTHFR levels or activity by specific gene mutations induces mild to moderate increases in plasma Hcy levels, which may relate to placental dysfunction early during pregnancy [23]. Taking this into perspective two experiments were carried out and presented in Chapter 5. Two SNPs of the MTHFR gene were analyzed (C667T and A1298C) in plasma (the first study) and in placenta (second study) of women complicated with PE. The first study found that the prevalence of the CC mutant genotype of the A1298C polymorphism was higher in plasma among PE women. This mutation among PE women was related to increased neck circumference (an indirect index of overweight/obesity) and higher Hcy levels. No differences were found regarding the prevalence of the C677T polymorphisms among cases and control. In the second study, the prevalence of the same polymorphisms was subsequently analyzed in placental material of PE cases compared to controls. Contrary to the first study, frequency of the TT mutant genotype of the C677T polymorphism was higher in the placenta of pregnancies complicated with PE. Both mutations have been related to higher Hcy content and subsequent development of PE via oxidative stress or endothelial dysfunction.

SUMMARY

To date the exact cause of PE is unknown. Despite this, studies have shown that PE is a multifactorial disease related to pregnancy which involves various pathways and mechanisms that are interconnected. The present thesis presents important data that highlight the fact the PE is not only a vascular disease with three interesting contributions: a) higher sFlt-1 found in the third trimester have lead others to explore its predictive utility for first trimester PE screening together with other biochemical and biophysical aspects; b) the altitude of residency seems to be a factor imposing differences in the levels of NO and CoQ₁₀; and c) our data confirm that endothelial dysfunction is also present in the fetal circulation.

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CHAPTER 7

Valorisation

PRE- ECLAMPSIA IS MORE THAN A VASCULAR DISEASE

The studies reported in the present thesis underline the importance of several markers and genetic aspects that evidence key issues in the pathogenesis of pre- eclampsia (PE) which could in the near future serve as predictors of the development of the disease.

RELEVANCE

Approximately 5 to 14% of all pregnancies may be complicated with PE. It is a serious issue occurring during women's reproductive years. Despite attempts at prevention or intervention it is still a leading cause of maternal and fetal morbidity and mortality in both developed and non developed countries. There is no treatment for this disorder other than the termination of pregnancy which increases the rate of iatrogenic preterm births. Much research in the past has mainly focused on epidemiological, clinical and preventive aspects; today biochemical and molecular aspects seem to gain relevance as once we identify who will develop PE, intervention can be instated early, and although cure might not be feasible, at least its negative impact can be ameliorated. Although there have been advances in prenatal and neonatal care, prediction of PE is still shadowed by the lack of a unique predictive biochemical or molecular marker which could: a) allow its early detection and management and b) aid in the selection of appropriate candidates for new therapeutical approaches.

INNOVATION

Currently, the cause of PE is unknown; however, what we do know is that early diagnosis of PE improves maternal and fetal outcome. This thesis provides important insights to the understanding of several pathogenic pathways in the development of PE. PE in Latin America is a serious problem as it causes not only increased maternal fetal morbidity and mortality yet it imposes an important and significant burden to the healthcare system. Poverty conditions observed in Latin America together with inadequate prenatal care seem to increase the negative impact that PE has over female health and the healthcare system. Despite the limitations of the poverty conditions found in our country the present thesis sought at providing important insights in the search of a unique or various cost effective markers required for the screening and early detection and intervention of the problem of women of any socio-economic condition. In this sense, this thesis is innovative in its conception, first because it provides evidence that endothelial dysfunction is also present in the fetal circulation. Such data is still scarce or non existent. Second, it provides insights on various markers that evidence several pathogenic pathways of PE, highlighting the effect of altitude of residency in

terms of NO and CoQ₁₀ levels. The studied markers can be eventually validated in the near future for the early screening of PE. In this sense, the data presented in Chapter 2 regarding sFlt-1 and its higher levels found in PE women in 2009 definitively have lead others to study and validate this marker as a first trimester PE screening analyte together with other biochemical (i.e. placental growth factor, beta-HCG, alpha-fetoprotein, inhibin) and biophysical (i.e. uterine artery resistance o pulsatility index) aspects. This speaks about the originality of the contribution of our data, and despite eventual limitations our approach provides the basis for future novel investigation.

ACTIVITIES/PRODUCTS

Despite the fact that the biochemical markers and genetics aspects reported in this investigation seem promising in the prediction of PE, issues still remain to be elucidated. Although sFlt-1 seems a promising predictive marker it still needs to be combined with other biochemical and physical aspects, therefore there is still an urgent need of finding a unique marker that can not only predict the disease yet define the one abnormality that is the cause of the disease and not the effect. The author would also like to highlight the important role that genetics and epigenetics may have in elucidating the cause of the disease. Future research should focus in the correlation between genetic abnormalities or phenotypes encountered in women with PE with biochemical abnormalities and clinical stages or outcome of the disease.

SCHEDULE AND IMPLEMENTATION

Further perspectives for the utility of the studied biochemical markers, especially sFlt-1 and genetic aspects seem not only promising yet intriguing. Indeed, the possibility to validate the effectiveness of an innovative novel therapeutical approach for PE based on biochemical and genetic markers instead of clinical criteria seems a major hot topic for the near future. Our data provides insights that seem to aid this promising perspective.

CHAPTER 8

Acknowledgments

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Curriculum Vitae

Peter Chedraui was born on August 3rd, 1965, in New York City. He grew up in Astoria Queens until the age of 10, when he moved to Guayaquil, Ecuador to complete primary and secondary school. After this, he obtained his medical and master degree in science at the Universidad Católica de Santiago de Guayaquil. Subsequently he completed an OB GYN residency program at the Enrique C. Sotomayor Hospital of Guayaquil, Ecuador.

After completing his residency he was awarded a Fellowship sponsored by FIGO and ACOG in Maternal Fetal Medicine at NYU Medical Center under the tutorship of Dr. Charles J. Lockwood. Since 2005 he has been Chief of the High Risk Pregnancy Labor and Delivery Unit at Sotomayor Hospital. Since 2002 he has been Director of the Institute of Biomedicine, of the Universidad Católica and Adjunct professor at the same University.

He is a member of several National and International Scientific Societies, with more than 130 peer-reviewed publications supporting his scientific research career. He is currently President of the Ecuadorian Climacteric and Menopause Society, Vice-President of the Latin American Association of Gynecological Endocrinology, Re-Elected Board Member of the International Menopause Society, Elected Board member of the International Society of Gynecological Endocrinology and Editorial member of various international peer reviewed journals devoted to women's health.

He has garnished multiple grants from the Universidad Católica, the pharmaceutical industry and various private foundations. To date he is actively serving as a research collaborator for several international joint research programs.

His primary research interests are genetic aspects of pre- eclampsia and preterm birth and their impact over adult female health such as the metabolic syndrome and cardiovascular risk. In addition he is interested in the epidemiology of the menopause and other female healthcare issues of mid-life.

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