Placenta 135 (2023) 1–6

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Preeclampsia association of placental nucleotide variations in *eNOS, VEGFA,* and *FLT-1* genes in Latin American pregnant women

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ARTICLE INFO

Keywords:
Preeclampsia
Placenta
Nucleotide variations
eNOS
VEGFA
FLT-1

ABSTRACT

Introduction: Preeclampsia is a leading cause of maternal and fetal morbidity in low- and middle-income countries, including those in Latin America. Placental vascular alterations are crucial in the pathophysiology of preeclampsia and few studies have evaluated nucleotide variations on genes associated with vascular regulation in the human placenta. This study aimed to evaluate whether placental nucleotide variations on *eNOS*, *VEGFA,* and *FLT-1* genes are more frequently associated with preeclampsia in the Latin American population.
Methods: This case-control study included placental tissue from 88 controls and 82 cases that were genotyped through Taqman probes for *eNOS*, *VEGFA,* and *FLT-1* genes. The intergroup comparisons were analyzed with the Mann-Whitney *U* test. Genotype and allele frequencies were compared by the X^2 test. The association between the nucleotide variants with preeclampsia was evaluated through logistic regression analysis.
Results: A significant association was observed for VEGFA SNV rs2010963 (OR 1.95; CI 95% 1.13–3.37), after adjusting for population substructure. The allele combination T, G, G, C, C, C (rs2070744, rs1799983, rs2010963, rs3025039, rs699947 and rs4769613 respectively), showed a negative association with preeclampsia (OR 0.08; CI 95% 0.01–0.93). results.
Discussion: Placental SNV rs2010963 in the *VEGFA* gene was a risk factor for preeclampsia, while the allele combination T, G, G, C, C, C may represent potential protective factors for preeclampsia within Latin American women.

1. Introduction

Preeclampsia is a pregnancy-related syndrome characterized by widespread endothelial dysfunction. Currently preeclampsia is diagnosed by new-onset hypertension and proteinuria and/or other end-organ dysfunction in the second half of pregnancy [1]. This syndrome is considered one of the leading causes of maternal and fetal morbidity and mortality in low- and middle-income countries including those in Latin

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https://doi.org/10.1016/j.placenta.2023.02.010
Received 12 September 2022; Received in revised form 6 February 2023; Accepted 24 February 2023
Available online 1 March 2023
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America [2]. Underlying mechanisms of preeclampsia development remain under investigation; despite the enormous efforts to understand the pathophysiology for this condition. Current understanding of the pathophysiology indicates that shallow trophoblast invasiveness results in a deficient remodeling of spiral arteries, which is associated with impaired placental vascular formation and function, leading to placental hypoxia or placental ischemia-reperfusion injury. Therefore, the injured placenta releases harmful factors, including antiangiogenic and pro-inflammatory molecules into the maternal circulation, favoring generalized maternal endothelial dysfunction [3].

Populational studies have provided evidence of genetic susceptibility to preeclampsia [4–10]. Frequently studied genes are those encoding for endothelial nitric oxide synthase (*eNOS*), vascular endothelial growth factor (*VEGFA*), and VEGF receptor type 1 (*VEGFR1* or *FLT1*). Proteins derived from these specific genes are involved in several biological functions, including vascular tone regulation [11,12], angiogenesis [13–16], inflammation [17], oxidative and nitrative stress [18,19], placentation [20,21], among others. Of importance, all these mechanisms are largely studied in the pathophysiology of preeclampsia. For instance, eNOS is responsible for the production of the vasodilator nitric oxide (NO), whose concentrations are decreased in preeclampsia [22,23]. Additionally, VEGFA a pro-survival endothelial cell factor responsive to hypoxia-mediated angiogenesis, vasculogenesis, spiral arteries remodeling, and trophoblast survival [14] is impaired in preeclampsia with reports showing either increased [24] or decreased [25] levels. In addition, VEGFA functions are exerted through the activation of a tyrosine kinase receptors family, including VEGFR-1, VEGFR-2, and VEGFR-3 [26]. The VEGFR-1 encoding gene *FLT-1* is overexpressed in chorionic villi under hypoxic conditions [27]; while Li et al. found high concentrations of the soluble isoform s*Flt-1* in placentas of preeclamptic patients [28].

Different studies have evaluated nucleotide variations in *eNOS, VEGFA,* and *FLT-1* genes and their relationship with preeclampsia, with no conclusive or even contradictory results. The most common variations evaluated so far for *eNOS* are 894G > T [Glu298Asp (rs1799983)], the promoter variation −786T > C (rs2070744), and the variable number of 27 bp tandem repeats (VNTR) present on intron 4 [29,30]. While for *VEGFA*, the promoter variation −2578 C > A (rs699947), −634 G > C (rs2010963) located in the 5′UTR [31] and 936 C > T (rs3025039) at 3′UTR [32]; whereas for *FLT1,* the extra genic variant rs4769613 (T > C) has been also evaluated in preeclampsia [33,34]. The genetic background of the different populations studied could explain the discrepancy observed in those reports. Also, most of those studies have been carried out in maternal blood samples, rather than in placenta samples.

The placenta is crucial in the pathophysiology of preeclampsia, and this tissue is particularly relevant because it contains genetic paternal components [35]. However, only a few studies have incorporated nucleotide variations analysis of vascular-related genes in this tissue. For instance, Keshavarzi et al. (2017) found that −2549 Del/Del and −634 CC genetic variations in *VEGFA* are associated with a high risk of preeclampsia in the Iranian population [36]. The same group found a strong correlation between −634 CC genotype with high *VEGFA* mRNA placental levels [37]. On the other side, McGinnis found an increase in maternal serum sFLT1 in the presence of fetal allele C for the rs4769613 variant in the *FLT1* gene [33], which was further confirmed by Kikas et al. [34]. Significantly, these studies are geographically limited, and may not be extrapolated to different populations.

In this study, we aimed to evaluate the single nucleotide variations (SNVs) rs2070744 and rs1799983 in the *eNOS* gene; rs699947, rs2010963, and rs3025039 in the *VEGFA* gene; and rs4769613 in *FLT1* gene in placentas from Latin American pregnant women. We also analyzed the potential association between these SNVs with preeclampsia.

2. Methods

2.1. Selection of patients

A case-control study was conducted. Cases included placentas from women affected with preeclampsia, and control group included placentas from healthy pregnant women. The samples were obtained from patients recruited in Hospitals from Chile (Hospital Clinico Herminda Martin de Chillan, Chillan, Chile), Colombia (Hospital Universitario San Ignacio, Bogotá, Colombia), and Mexico (Hospital Materno-Infantil de la Ciudad de Durango, Durango, Mexico). All participants are self-declared mestizo. The research was carried out by the principles expressed in the Declaration of Helsinki and under the authorization of the respective Ethical Review Boards. All participants gave their informed consent before sample collection.

Women with preeclampsia were diagnosed, according to the International Society for Studies on Hypertension in Pregnancy (ISSHP) guidelines in 2014 [38]. Pregnancies resolved by vaginal delivery or cesarean sections were included. Patients with concurrent diseases were excluded. Parameters such as maternal age, weight before pregnancy, height, pregnancy resolution age, and sex and weight of the newborn, were registered.

Trained personnel obtained placental samples in the three participants' Hospitals. Samples were stored in the respective laboratory until use.

The sample size was calculated to have an odds ratio between 1 and 2, with a statistical power of 95%, a type 1 error level of 0.05, a minor allele frequency of 0.24, and a disease prevalence of 8%, this resulted in a minimal sample size of 58 individuals per study group. Considering a potential sample loss of 15% during processing, a final sample size of 68 individuals per group was calculated [39]. Matched parameters included gestational age at delivery, maternal age, sex of the newborn, year of delivery, and same reference center.

2.2. Placenta DNA isolation

Four small pieces of maternal-face fresh placentae collected no longer than 30 min after delivery were obtained and washed immediately in cold 1% phosphate buffer solution (PBS). The tissues were collected in RNA later™ and frozen. The DNA was extracted in each center using a standardized technical approach based on the modified rapid salt-extraction method [40], and after DNA isolation samples were stored at −20 °C until further processing. Three micrograms of DNA were embedded in Whatman® filter paper and shipped to the National Polytechnic Institute in Durango Mexico for genotyping. Recovery of DNA from Whatman filter paper was 75 ± 2%.

2.3. Genotyping analysis

Genotyping was carried out using a quantitative real time PCR (Q-PCR) system StepOne™ (Applied Biosystems®, Carlsbad, CA, USA) with the following Taqman™ probes: *eNOS* [rs2070744 (C_15903863_10) and rs1799983 (C_3219460_20)]; *VEGFA* [rs699947 (C_8311602_10), rs2010963 (C_8311614_10) and rs3025039 (C_16198795_10)]; and *FLT-1* [rs4769613 (C_32231378_20)].

The reaction conditions were as follows: one cycle of initial denaturing at 95 °C/10 min followed by 42 cycles of denaturing (95 °C/15 s), annealing (60 °C/1 min), and extension (60 °C/30 s). The reactions were performed in triplicate by the same trained personnel.

2.4. Statistical analysis

Data are presented as median (25th and 75th percentiles) due to the non-Gaussian distribution of data. Statistical differences between numerical variables were analyzed using Mann Whitney *U* test, while Fisher's exact test was used for categorical variables. The frequencies of

genotypes were obtained by direct count and Hardy-Weinberg equilibrium (HWE) was calculated through the X^2 goodness-of-fit statistic. Differences in genotype and allele frequencies were evaluated through the X^2 test. Logistic regression analysis was used to evaluate the association between nucleotide variations and preeclampsia.

The statistical software SNPstats [41], and Statistical Package for Social Sciences (SPSS) software V 15.0 were used in the analysis. $P < 0.05$ was considered statistically significant.

3. Results

In this case-control study, 170 placental samples were included. Of these, 88 (51.8%) belonged to the control group whereas 82 (48.2%) were classified as cases. The maternal age, weight before pregnancy, height, pregnancy resolution age, and sex of newborn showed no significant differences between groups (Table 1). However, newborns in the cases group were significantly smaller than those in the control group ($p < 0.01$).

The allele and genotype frequencies of the studied nucleotide variants are shown in Table 2. The results revealed significantly higher frequency of genotypes (TC) (*eNOS*, rs2070744, $p = 0.008$), (GG) (*VEGFA*, rs2010963, $p = 0.04$) and (AA) (*VEGFA*, rs699947, $p = 0.003$) in the control group compared to cases. The minor allele (C) was significantly more frequent (*VEGFA*, rs2010963, $p = 0.027$) in the cases group. The 6 studied nucleotide variations were found in HWE ($p > 0.05$) in the whole population.

The logistic regression analysis to associate nucleotide variation with preeclampsia revealed a negative significant association of *eNOS* SNV rs2070744 (OR 0.43; CI 95% 0.22–0.86) under a codominant inheritance model, while *VEGFA* SNV rs699947 (OR 0.17; CI 95% 0.05–0.61) under a recessive inheritance model. Conversely, a significant association with preeclampsia (OR 1.96; CI 95% 1.03–3.74) was observed for *VEGFA* SNV rs2010963 under a dominant inheritance model (Table 3). Under an additive logistic regression model adjusting for country in which sample was collected, the *VEGFA* SNV rs2010963 remain significantly associated with preeclampsia (OR 1.95; CI 95% 1.13–3.37), meanwhile the association observed for SNVs rs2070744 and rs699947 was lost.

The analysis of haplotypes for *eNOS* and *VEGFA* revealed no association with preeclampsia. However, the combination of alleles T, G, G, C, C, C for SNVs rs2070744 and rs1799983 (*eNOS*), rs2010963, rs3025039 and rs699947 (*VEGFA*), and rs4769613 (*FLT-1*) respectively, showed a negative association with preeclampsia (OR 0.08; CI 95% 0.01–0.93). The frequency of such a combination was 9.56%.

4. Discussion

We report that after adjusting for population substructure, the placental SNV rs2010963 in the *VEGFA* gene is associated with preeclampsia in pregnant women from three Latin American countries. Conversely, a potential protective effect was observed in the presence of allele combinations T, G, G, C, C, C derived from the six analyzed SNVs.

Table 1
Clinical features of the studied subjects.

	Cases n = 82	Controls n = 88	*p*-value[a]
	Median (min-max)	Median (min-max)	
Chronological age (years)	29.0 (15–42)	27.5 (15–41)	0.456
Gestational age (weeks)	37 (31–40)	39 (34–41.2)	0.074
Systolic pressure (mmHg)	158 (140–185)	115 (91–131)	<0.001
Diastolic pressure (mmHg)	100 (76–125)	70 (55–82)	<0.001
Pregestational weight (kg)	79.0 (45–120)	69.0 (51–102)	0.082
Height (cm)	160 (149–170)	160 (146–170)	0.830
Newborn weight (g)	2540 (1315–3625)	3168.5 (2275–4915)	<0.001
Newborn gender Female/Male n (%)	43/39 (52.4/47.6)	43/45 (48.8/51.1)	0.151°

[a] Mann-Whitney *U* test, °Fisher exact test.

Table 2
Genotype and allele frequencies of the studied nucleotide variants.

		Controls n = 88 n (f)	Cases n = 82 n (f)	*p*-value
eNOS	Genotypes			
rs2070744	TT	42 (0.52)	50 (0.67)	0.06
T > C	**TC**	**37 (0.46)**	**19 (0.25)**	**0.008**
	CC	2 (0.02)	6 (0.08)	0.155
	Alleles			
	T	121 (0.75)	119 (0.79)	0.331
	C	41 (0.25)	31 (0.21)	
eNOS	Genotypes			
rs1799983	GG	29 (0.39)	39 (0.52)	0.101
G > T	GT	42 (0.56)	32 (0.43)	0.102
	TT	4 (0.05)	4 (0.05)	1
	Alleles			
	G	100 (0.67)	110 (0.73)	0.208
	T	50 (0.33)	40 (0.27)	
VEGFA	Genotypes			
rs2010963	**GG**	**47 (0.57)**	**28 (0.40)**	**0.040**
G > C	GC	33 (0.40)	35 (0.50)	0.204
	CC	3 (0.03)	7 (0.10)	0.187
	Alleles			
	G	**127 (0.77)**	**91 (0.65)**	**0.027**
	C	**39 (0.23)**	**49 (0.35)**	
VEGFA	Genotypes			
rs3025039	CC	43 (0.58)	34 (0.49)	0.290
C > T	CT	30 (0.41)	31 (0.45)	0.596
	TT	1 (0.01)	4 (0.06)	0.197
	Alleles			
	C	116 (0.78)	99 (0.72)	0.194
	T	32 (0.22)	39 (0.28)	
***VEGFA* rs699947**	Genotypes			
C > A	CC	37 (0.44)	34 (0.47)	0.691
	CA	30 (0.36)	35 (0.49)	0.103
	AA	**17 (0.20)**	**3 (0.04)**	**0.003**
	Alleles			
	C	104 (0.62)	103 (0.72)	0.073
	A	64 (0.38)	41 (0.28)	
***FLT1* rs4769613**	Genotypes			
T > C	TT	32 (0.37)	28 (0.36)	0.950
	TC	47 (0.53)	37 (0.47)	0.442
	CC	9 (0.10)	13 (0.17)	0.222
	Alleles			
	T	111 (0.63)	93 (0.60)	0.519
	C	65 (0.37)	63 (0.40)	

n = Individuals; value (f) is frequency. Significant differences are remarked in bold. X^2 test.

Table 3
Models of inheritance of the associated SNVs.

Crude analysis				
SNV	Model	Genotype	OR (95% CI)	*p*-value
eNOS rs2070744 T > C	Codominant	T/C	0.43 (0.22–0.86)	**0.015**
VEGFA rs2010963 G > C	Dominant	G/C–C/C	1.96 (1.03–3.74)	**0.040**
VEGFA rs699947 C > A	Recessive	A/A	0.17 (0.05–0.61)	**0.002**
Adjusted by population substructure analysis				
VEGFA rs2010963 G > C	Additive	G/C	1.95 (1.13–3.37)	**0.016**

OR, odds ratio. Logistic regression analysis.

Therefore, nucleotide variations detected in the placenta of women with preeclampsia may constitute a biomarker for vascular placental alterations.

4.1. Results in the pathophysiological perspective

The genes *eNOS, VEGFA, and FLT-1* are all related to preeclampsia development. In 2012, Luizon et al., demonstrated the epistatic interaction between *eNOS, VEGFA,* and metalloproteinase 9 (*MMP-9)*, revealing that different genotype combinations on the three genes are associated with preeclampsia [42]. In the same study, the individual analysis of SNVs revealed no association with the disease. In the present study, the SNV rs2010963 in the *VEGFA* gene almost doubles the risk for preeclampsia.

A growing body of evidence [29,43–45], including some studies conducted with Latin American women with preeclampsia [23,29,30], reports similar SNVs for the target genes evaluated in the present study. Unfortunately, most of them are focused on the analysis of maternal samples, including plasma or circulating cells (i.e., monocytes), and they are not directly comparable with the present report, conducted on placental tissue. Analysis of placenta vascular genes may correspond with placental blood vessel alterations, either in the formation [29,46,47] or in the function [43,48–51] observed in women with preeclampsia.

In fact, few studies identifying SNVs have been carried out in placental tissue. In 2017, Keshavarzi et al., identified in Iranian population a couple of nucleotide variations associated with preeclampsia in the VEGFA gene: 2549 Del/Del and −634 CC [36], being the last SNV coincident with our results. The same study revealed an even stronger association of −634CC genotype with severe preeclampsia [36]. Unfortunately, considering the sample size evaluated in the present study, the stratification by preeclampsia severity was not performed.

Nevertheless, Keshavarzi et al. evaluated the same nucleotide variations and expression of *VEGFA* mRNA in placental tissue and found that −634CC genotype was associated with high expression of the gene [37]. Despite, we did not measure the transcript levels of any of the analyzed genes, the result obtained by Keshavarzi could lead us to suppose that the levels of mRNA would be elevated in the placentas of patients with preeclampsia.

A meta-analysis including 11 case-control studies was performed by Cheng et al., who showed association of SNVs +936C/T (rs3025039) and −634G/C (rs2010963) with preeclampsia and lack of association with SNVs −2578C/A (rs699947) and −1154G/A (rs1570360) [52]. The association with preeclampsia was also observed in the present study for rs2010963, but not for rs3025039. A recent meta-analysis evaluating SNVs +936C/T, −634G/C, −2578C/A, and −1154G/A in 23 studies, revealed an association of +936C/T (rs3025039) with preeclampsia in Europeans and Asians populations, meanwhile −634G/C (rs2010963) was associated with the disease only in Europeans populations [53]. It is remarkable that rs2010963 is consistently associated with preeclampsia in both meta-analyses, agreeing with the results of the present study. On the other side, inter ethnicity are important, and could explain the differences between these studies and our report.

In the present study, we did not find an association between the variant rs4769613 in the *FLT1* gene with preeclampsia. This result is intriguing since this variant was associated with preeclampsia in a larger population study including offspring of women with preeclampsia [33]. The authors recognized the occurrence of this SNV as being related to a higher concentration of sFLT1 in maternal serum, within the control population. The lack of association observed in the present study could result of interethnic differences because McGinnis evaluated Caucasian populations meanwhile; we analyzed mestizo populations from Latin America. Kikas et al., including an Estonian population replicated the McGinnis's results regarding the SNV rs4769613. These authors found an association of preeclampsia with placental but not maternal or paternal derived allele C [34]. In addition, Ohwaky et al., evaluated in a Japanese population the variant rs4769613 and found no difference between women with or without preeclampsia [54], reinforcing our findings and the importance of interethnic differences of similar studies. One of the strengths of our study is that it is the first to evaluate at once several SNV in three functionally related genes in placental tissue, observed in three different populations from Latin America.

The haplotypes for *eNOS* and *VEGFA* genes showed no association with preeclampsia in the present study. On the other side, the allele combination T, G, G, C, C, C [*eNOS* (rs2070744 and rs1799983), *VEGFA* (rs2010963, rs3025039 and rs699947) and *FLT1* (rs4769613)] respectively, revealed a negative association with the disease, suggesting its potentially protective nature. It is important to note that the first five are all wild alleles, while the latest one in *FLT1* gene is the mutant type allele.

Some limitations in the present study deserve to be mentioned. For instance, we cannot discard heterogeneity in the three analyzed populations, as we did not perform genetic ancestry evaluations. However, by self-report, all volunteers self-declared to be mestizo. In this regards, Mendoza-Revilla et al., have analyzed European, Native-American and African ancestry in people from Chile, Colombia and Mexico showing a similar pattern in the distribution of ancestry. Thus, Colombian presented the highest European ancestry, followed by Chile and Mexico; Mexican presents a higher Native-American ancestry, followed by Chile and Colombia. Among the three countries, Colombia has the highest African ancestry, followed by Mexico and Chile [55]. Also, future studies in our population should include analysis of SNVs considering severity of preeclampsia, and importantly, correlate those analyses with the presence of vascular alterations in the placenta. Furthermore, we also encourage future studies that correlate placental SNVs with the respective placental expression of each gene and/or serum analysis seeking for biomarkers of placenta dysfunction. Finally, genotyping analysis in the placenta will help us to better understand the pathophysiology of preeclampsia and perhaps future cardiovascular risk in the offspring. But these hypotheses need to be tested.

In conclusion, in Latin American samples after adjusted analysis, the placental SNV rs2010963 in VEGFA gene represents a risk factor for preeclampsia development. Conversely the allele combination T, G, G, C, C, C [eNOS (rs2070744 and rs1799983), VEGFA (rs2010963, rs3025039 and rs699947) and FLT1 (rs4769613)], respectively are potentially protective for preeclampsia.

Author contributions

CG-H and CE conceptualized the study. AMS write the manuscript. CG-H and MS-M conducted the statistical analyses. JB-Z and RB-C performed most of the experiments in this manuscript. CG-H, MS-M, CE, RG, PA and JB were responsible for patient recruitment. CE, CG-H, AD and FRG edited the manuscript. All co-authors approved the final version of this manuscript.

Declaration of competing interest

The authors do not have any conflict of interest to declare.

Acknowledgment

The authors would like to thank the researchers belonging to RIVATREM, and clinical staff of Hospital Clinico Herminda Martin de Chillan (Chile), Hospital Universitario San Ignacio (Bogotá, Colombia), and Hospital Materno-Infantil de la Ciudad de Durango (Durango, México) for their valuable contributions during this project. Carlos Escudero is funded by Fondecyt 1200250 (Chile). Carlos Galaviz-Hernandez and Martha Sosa-Macías are funded by Secretaria de Investigación y posgrado IPN (SIP-20221632 and SIP-20221634).

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