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ORIGINAL ARTICLE

Evaluation of Placental VEGFA mRNA Expression in Preeclampsia: A Case Control Study

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Abstract

Objective The aim of our case–control study was to determine expression of VEGFA mRNA in placentae of preeclamsia (PE) versus uncomplicated pregnancy to further clarify its differential expression in pregnancy hypertensive disorders.

Study Design The PE group was subdivided into severe and non-severe; those with or without HELLP syndrome and placental VEGFA characteristics were compared for these cohorts. Additionally, the neonatal and maternal outcomes were recorded. The quantification of placental VEGFA was done using quantitative real-time PCR and results were expressed as fold change.

Results Out of 42 PE cases, 23 (55%) were non-severe and 19 cases (45%) were severe PE. Out of 19 severe PE patients, 8 (42%) were HELLP syndrome (complete HELLP) and remaining 11 (58%) were non-HELLP severe PE. Compared to controls, the true fold change in PE,

HELLP, non-HELLP, severe PE, non-severe PE was − 2.186, − 13.333, − 6.698, − 8.950 and 1.466, respectively.

Conclusions Our results showed a lowered VEGFA expression in PE placentae compared to uncomplicated controls. The finding of initial increase of VEGFA in non-severe PE and subsequent marked lowering in HELLP strengthens the existing hypothesis of decompensated VEGF being a major role player in PE.

Keywords VEGF · Placenta · Preclampsia

Introduction

Preeclampsia (PE), which is diagnosed by hypertension and proteinuria after 20 weeks of gestation, is one of the major causes of fetomaternal morbidity and mortality [1]. The exact pathophysiology of PE remains still unknown, but is closely related to development of placenta [2]. More recently, the angiogenic vascular endothelial growth factors (VEGF) family including VEGFA-D and placental growth factor (PIGF) has been extensively studied for their role in PE [2, 3]. Of these, VEGFA and PIGF acting through their receptors VEGFR1 (FLT1—Fms-like tyrosine kinase receptor-1), VEGFR2 (Kinase insert domain receptor—KDR) are considered most important in regulating early placental vascular development during pregnancy [2, 3]. VEGFA, PIGF, FLT1 and KDR are expressed in villous and extravillous trophoblasts, villous vascular endothelium as well as in natural killer cells and their level of expression is known to be altered in PE.

These properties of VEGF have led to its investigation as a potential pathophysiological molecule in PE. The majority of research studies have compared serum VEGF levels in the maternal circulation in PE pregnancies versus uncomplicated pregnancies [2, 4]. Another area of investigation is quantification of VEGF and its receptors within placenta. However, there is substantial discrepancy in the literature concerning levels of placental VEGF in PE pregnancies [2, 5–11]. Placental VEGF mRNA levels have been reported to be decreased, increased or unchanged in PE cases versus uncomplicated controls. We, therefore, undertook this study to determine the expression of VEGFA mRNA in placentae of PE versus uncomplicated pregnancy in order to clarify the various discrepancies and gain further evidence for its role in PE. The PE group was subdivided into severe and non-severe, those with or without HELLP syndrome and placental VEGFA characteristics were compared for these cohorts. Additionally, the neonatal and maternal outcomes were recorded.

Materials and Methods

The observation case–control study was conducted in tertiary health care institution in a low income Indian subcontinent country in joint collaboration with Department of Obstetrics and Gynecology and Department of Biochemistry between November 2015 and April 2017. Ethical clearance (dated 21 October 2015) and informed patient consent were obtained for this case–control study.

Sample Size Calculation

Considering a decrease of 53% [5] in VEGF mRNA expression in placenta of cases of PE compared to control, at $\alpha = 5\%$ and power of 90% a sample of 15 cases was required in each group. Considering the variation in expression of VEGF mRNA in Indian population, we took 42 subjects in each group.

Our inclusion criteria for cases were PE women [Group 1, cases] (PE, defined as blood pressure $\geq$ 140/90 mm Hg after 20 weeks of gestation with proteinuria in a previously normotensive female. Proteinuria was defined as 24 h urine protein $\geq$ 300 mg/24 h) [1].

Our exclusion criteria were women with gestational hypertension, chronic hypertension, pregnancy with known collagen vascular disease, chronic liver, renal disease, Rh isoimmunization, spontaneous preterm labor, diabetic or gestational diabetes. Patients with anomalous fetus and multiple gestations were also excluded.

An equal number of normotensive women with uncomplicated pregnancy at delivery [Group 2, controls] matched for age, socioeconomic status, religion and occupation were also enrolled (Fig. 1). All cases were investigated for PE which included laboratory and fundus examination. Mode of delivery was decided on case to case basis as per hospital protocol. At the time of delivery, weight of the baby was noted. Neonatal outcome was stated in terms of APGAR score at 0, 5 and 10 min. Primary outcome was comparison of placental VEGF mRNA expression in PE patients and normal controls. Secondary outcome was comparison of placental VEGF mRNA expressions in severe and non-severe PE.

Immediately after the delivery, a placental piece of 5 × 5 cm size was dissected out from maternal surface of peripheral placenta and transferred for extraction of VEGFA mRNA evaluation. Placental tissue with macroscopic evident hemorrhage, necrosis, or calcification was excluded. Briefly, the method of placental processing included tissue homogenation, extraction of mRNA, formation of cDNA from RNA by reverse transcription, quantification of VEGF by quantitative real time PCR

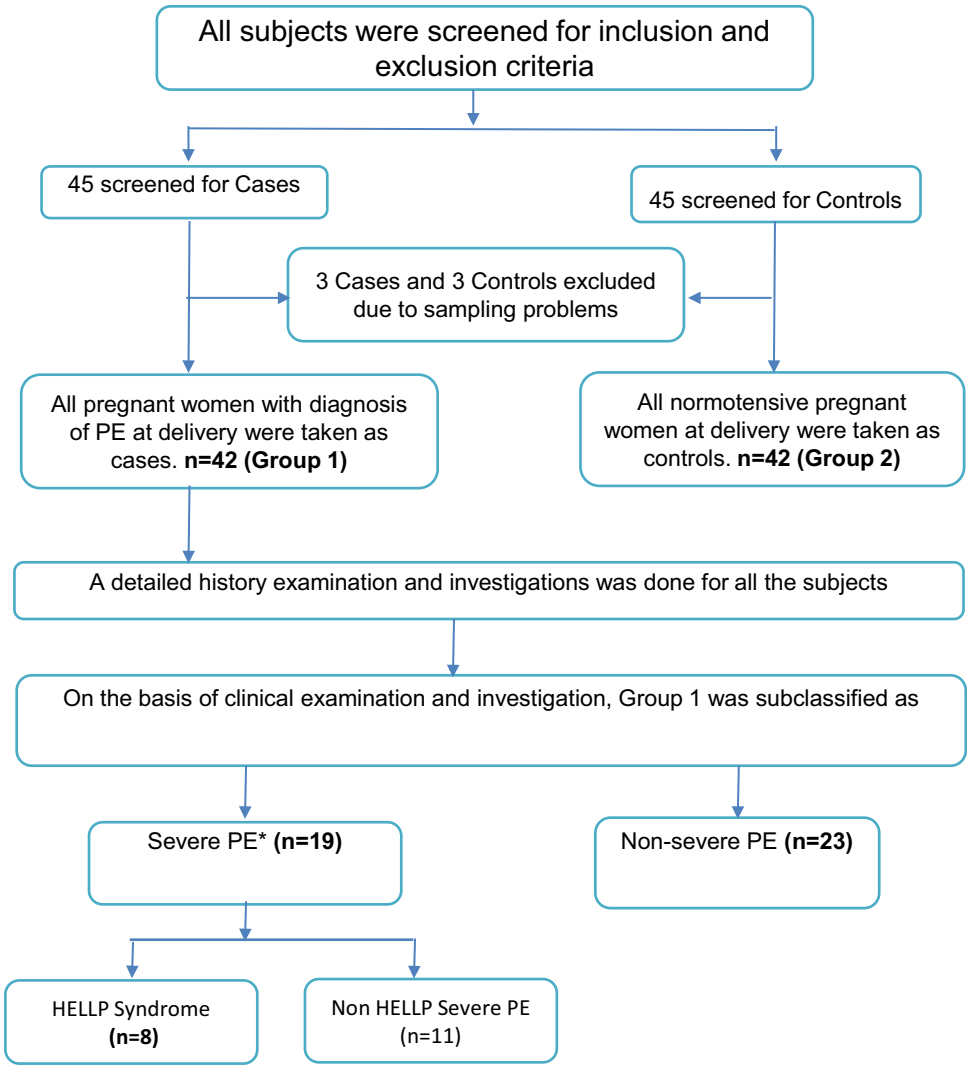

Fig. 1 Study flow diagram depicting methodology. *Severe PE [1]: Diastolic BP ≥ 110 mm Hg; systolic BP ≥ 160 mm Hg; headache; visual disturbances; upper abdominal pain; oliguria; elevated serum creatinine; thrombocytopenia (< 100,000/μl); serum transaminase elevation; pulmonary edema

(qRT PCR) and its analysis. The primer sequences used are given in Table 1.

The results were scrutinized as per fold change (FC) analysis for evaluating differential expression of genes. In this, gene expression normalization was done using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) constitutive genes by determining delta Ct (cycle threshold; ΔCt). Ct values are inversely proportional to the amount of target nucleic acid in the sample, i.e., the lower the value of Ct, the higher the amount of target nucleic acid in the sample. Delta Ct (ΔCt) is the difference of average Ct of target gene (VEGF gene) and constitutive gene (GAPDH gene): average Ct target–average Ct normalizes. Using this formula, the difference of mean Ct values of control and cases was determined, which is delta delta Ct ($\Delta\Delta Ct$) = $\Delta Ct_{group\ 2} - \Delta Ct_{group\ 1}$. Fold change (FC) compares the expression of genes between cases (Group 1) and controls (Group 2) by the following:

$FC = 2^{\Delta\Delta Ct}$

If FC > 1, the True FC = FC and implies upregulation of gene.

If FC < 1, then True FC = − 1/FC and implies downregulation of gene.

Thus, the True FC reflects the number of times the gene expression was upregulated or downregulated.

Statistical Method Used

Data were analyzed using Microsoft Excel (version 2010) and statistical software SPSS (version 20.0). p value < 0.05 was considered as significant.

Unpaired Student's t test and Chi square/Fisher's exact test were applied to compare all sociodemographic characteristics, clinical profile, maternal, neonatal outcome and VEGF gene expression in cases and controls according to the data being quantitative and qualitative, respectively. Results were expressed in terms of fold change.

Table 1 Primer sequences of GAPDH and VEGF genes

Genes	Primer sequences	Amplicon size (bp)
GAPDH-FP	5′-CCAAGGTCATCCATGAGA-3′	381
GAPDH-RP	5′-TGTTGAAGTCAGAGGAGA-3′	
VEGFA-FP	5′-CTGGAGTGTGTGCCCACTGA-3′	96
VEGFA-RP	5′-TCCTATGTGCTGGCCTTGGT-3′	

FP forward primer, *RP* reverse primer

Table 2 Comparison of obstetrical and neonatal parameters in two study groups

Obstetrical and neonatal parameters	Group 1 Cases (n = 42) mean ± SD	Group 2 Controls (n = 42) mean ± SD	p value*
Age	26.19 ± 3.37	26.17 ± 3.46	0.975
Parity n (%)			0.105
Primigravida	15 (36)	7 (17)	
Multigravida	27 (64)	35 (83)	
POG at the time of delivery (mean ± SD)	37.98 ± 1.07	38.98 ± 1.24	0.003*
POG ≥ 38 weeks n (%)	24 (57)	37 (88)	
POG 37–38 weeks	18 (43)	5 (12)	
Labor n (%)			
Spontaneous	9 (21)	32 (76)	
Induction of labor	27 (64)	10 (24)	
Cesarean without induction/labor	6 (14)	0	
Mode of delivery n (%)			
Vaginal	36 (86)	38 (90)	
Instrumental vaginal delivery	0 (0)	2 (5)	
Cesarean section	6 (14)	2 (5)	
Birth weight (kg) (mean ± SD)	2.44 ± 0.33	2.70 ± 0.31	0.000*

POG period of gestation

*p value ≤ 0.05 has been considered as significant; Fisher exact test

Results

Out of 42 Group 1 cases, 23 (55%) were non-severe PE and 19 (45%) severe PE. Out of 19 severe PE patients, 8 (42%) were HELLP syndrome (complete HELLP) and remaining 11 (58%) were non-HELLP severe PE (Fig. 1). The obstetrical and neonatal parameters in Group 1 and 2 are given in Table 2. The mean gestation age at delivery was 37.98 weeks in Group 1 versus 38.98 weeks in Group 2. In Group 1, induction of labor was required in 64% (n = 27); cesarean section without induction in 14% (n = 6) and rest (n = 9) had spontaneous labor. In control Group 2, induction of labor was needed in 24% (n = 10) and spontaneous labor occurred in 76% (n = 32). The commonest mode of delivery was vaginal (86%) in Group 1. For Group 2, it was 90% for vaginal and 5% each for instrumental vaginal delivery and cesarean sections (Table 2). None of the patients complained blurring of vision or epigastric pain. One patient had renal failure with HELLP syndrome. The birth weight of neonates born to severe PE women (2.64 kg ± 0.20) was significantly low as compared to non-severe PE women (2.19 kg ± 0.29) (p = 0.000*).

Comparison of Placental VEGFA Between Group 1 and Group 2

The mean value of VEGFA ΔCt was higher in Group 1 (18.73 ± 3.71; range, 11.44–27.73) as compared to Group 2 (17.60 ± 4.04; range, 10.24–23.79) indicating decreased mRNA expression of VEGFA gene in PE (p = .186). On calculating true fold change [$-1/(2^{\Delta Ct\ group\ 2\ -\ \Delta Ct\ group\ 1})$- $= -1/2^{-1.128}$], the expression of VEGFA gene was found − 2.186 fold (lower) in Group 1 as compared to Group 2.

Table 3 Comparison of VEGFA expression in subgroups of PE with controls

Parameter	Subgroups of PE (Group 1)	Controls (Group 2) (n = 42)	p value*
HELLP severe (n = 8)			
VEGFA Delta Ct			
Range	16.38–27.73	10.24–23.79	0.019*
Mean ± SD	21.34 ± 3.80	17.60 ± 4.04	
Non-HELLP severe (n = 11)			
VEGFA Delta Ct			
Range	13.89–24.75	10.24–23.79	0.045*
Mean ± SD	20.34 ± 3.54	17.60 ± 4.04	
Severe PE (n = 19)			
VEGFA Delta Ct			
Range	13.89–27.73	10.24–23.79	0.002*
Mean ± SD	20.76 ± 3.58	17.60 ± 4.04	
Non-severe PE (n = 23)			
VEGFA Delta Ct			
Range	11.44–22.25	10.24–23.79	0.566
Mean ± SD	17.05 ± 2.94	17.60 ± 4.04	

*p value ≤ 0.05 has been considered as significant; unpaired t test

Comparison of Placental VEGFA Subgroups

The mean value of VEGFA ΔCt was significantly higher in severe PE (n = 19) (20.76 ± 3.58; range 13.89–27.73) compared to non-severe PE (n = 23) (17.05 ± 2.94; range 11.44–22.25) cases (p = .001). On calculating true fold change [$-1/2^{-3.713}$], the expression of VEGFA gene was found − 13.118 fold (lower) in severe cases.

The mean ΔCt value of VEGFA increased in both HELLP and non-HELLP in severe PE category as compared to controls (Table 3). Results were statistically significant in both HELLP (p = 0.019) and non-HELLP (p = 0.045) when compared to controls. True fold change for HELLP syndrome and controls was − 13.333 (lower) and between non-HELLP and controls was − 6.698 (lower).

Results were statistically significant for ΔCt value of VEGFA in severe PE (p = 0.002) compared to controls. When true fold change between two was calculated, it was − 8.950 (lower). In the non-severe PE versus control, the true fold change was positive with value 1.466 (increased).

Comparison of VEGFA placental expression of HELLP and non-HELLP cases with non-severe PE revealed significant differences (Table 4). True fold change for HELLP syndrome and non-severe PE was − 19.542 (lower). True fold change for non-HELLP syndrome and non-severe PE was − 9.817 (lower).

Discussion

Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen and mediates vasculogenesis, angiogenesis, microvascular permeability and vasodilatation during establishment of maternal fetoplacental circulation [11]. Hypoxia is a potent stimulus for induction of

Table 4 Comparison of VEGFA expression of HELLP and non-HELLP cases with non-severe PE

Parameter	Type of severe PE (n = 19)	Non-severe PE (n = 23)	p value*
HELLP (n = 8)			
VEGFA Delta Ct			
Range	16.38–27.73	11.44–22.25	0.003*
Mean ± SD	21.34 ± 3.80	17.05 ± 2.94	
Non-HELLP (n = 11)			
VEGFA Delta Ct			
Range	13.89–24.75	11.44–22.25	0.007*
Mean ± SD	20.34 ± 3.54	17.05 ± 2.94	

*p value ≤ 0.05 has been considered as significant; unpaired t test

VEGF gene expression. VEGF and other factors are also likely to be involved in the regulation of the trophoblast invasion, proliferation and differentiation [11]. In the human placenta, this factor has chiefly been identified in the villous cytotrophoblast in the first trimester and in the syncytiotrophoblast and extravillous trophoblast in the term placenta. [11]

Estimation of plasma or serum concentration of VEGF and its expression in the placental tissue of women with pregnancies complicated by hypertensive disorders has been a topic of active research in view of its possible therapeutic implications. But the results have conflicting and controversial [2, 5–11]. Cooper et al. obtained biopsies of cesarean delivered placenta in 23 cases of PE pregnancies and compared them with 20 appropriately matched women with uncomplicated pregnancies [6]. They found that levels of VEGF mRNA were significantly lower in the PE women compared with the control women ($p < 0.023$) [6]. Kim et al. [7] also reported decreased expressions of VEGF in both level of mRNA and protein in third trimester placental tissue of PE patients ($n = 20$) compared with an equal number of normotensive controls ($p < 0.05$). Andraweera et al. [5] compared mRNA placental expression of VEGFA in placental tissue obtained at delivery from PE ($n = 18$), gestational hypertension ($n = 15$) and uncomplicated pregnancy ($n = 30$). Compared to placental mRNA from uncomplicated pregnancies, VEGFA were reduced in PE ($p = 0.006$) and gestational hypertension ($p < 0.001$) placentae [5].

In contrast, some studies have showed an increased VEGFA expression in placental tissues [8, 9]. The Lee et al.'s study with aim of investigating cytokine and oxidation related genes found upregulation of VEGFA mRNA in 13 PE placentae [8]. Similarly, Chung et al. showed that total VEGF mRNA expression was increased 2.8-fold ($p < 0.05$) in PE versus normal placenta [9]. The study also quantified different VEGF isoforms (VEGF 121, 165, and 189) in PE versus normal placentas. Compared with uncomplicated pregnancy, the placental mRNA levels of three VEGF isoforms in PE were elevated ($p < 0.05$) 1.8, 1.9, and 1.7 fold, respectively, for VEGF 189, 165, and 121, as compared with normal placentas.

A few studies have also reported unaltered expression of VEGFA in placental tissues in PE women [10, 11]. Ranheim et al. reported that there were no statistically significant differences in expression of VEGF in mRNA levels for either the decidua basalis or placental tissues in a study conducted in 25 PE and 19 uneventful pregnancies [10]. Sgambati et al. reported that in cases of PE, the levels of VEGF mRNA were the same as the control group [11]. Their study group of placentas from women with gestational hypertension ($n = 20$), PE ($n = 20$) and PE with HELLP syndrome ($n = 20$) and from normotensive control group women ($n = 20$) showed the VEGF-positive components of all the pathological groups having a higher intensity of reactivity with respect to that of the control.

The discrepant results observed among these studies may be due to the differences in sample size, phenotypic classification of PE chosen, the timing of placental tissue sampling, the method of VEGF quantification and outcome depiction and the references taken for comparison [2, 3]. There are many genetic and environmental factors that may further alter gene expression of PE. Furthermore, the pathophysiology of VEGF expression in PE is far from complete decipherment with numerous postulates available for explaining different obtained results [5, 6, 11]. As such, we considered it worthwhile to plan our study with a case–control model in a different population to add evidence to the existing literature.

Our findings revealed that the placental mRNA VEGFA expression was lowered in PE cases compared to normotensive controls. Decrease was much severe in HELLP and severe PE category (Fig. 2). Further, we found increased expression of VEGF mRNA in non-severe PE patient as compared to controls. Our findings support hypothesis by Sgambati et al. that the VEGF levels might be related to different degrees of clinical severity of pregnancy hypertensive disorders [11]. It is known that hypoxic placentae from pregnancies with hypertensive disorders stimulate the VEGF production. Therefore, in lesser clinical presentations (non-severe PE), there is an *initial* compensatory increase of placental VEGF expression in attempting to restore the blood flow towards normal. With progression of hypertensive pathology, more dysfunctional and damaged placental tissue results in lower VEGF despite compensatory attempts. In pregnancies with HELLP syndrome, one of the most severe forms of PE, VEGF level stood lowest compared to controls (approx. − 13 times) (Fig. 2).

We could not study concomitant placental VEGF receptors expression due to financial limitations. Immunohistochemistry, estimation of proteins using ELISA and placental structure were not a part of our study. Serum and neonatal cord blood VEGF levels were not considered in our study. The strengths were a case–control model study in a previously uninvestigated geographic location of the pregnancy cohort, i.e., population from Indian subcontinent. Our study focused on a well-defined PE group excluding gestational hypertension and eclampsia cases to reduce ambiguity of pregnancy related hypertensive case definitions. We used a bigger sample size ($n = 42$) compared to previous studies and quantitative real time PCR (qRT PCR) for quantification of differential expression of genes for our study. Our results showed a lowered VEGFA expression in PE placentae similar to studies by Cooper, Kim and Andraweera et al. [5–7]. The

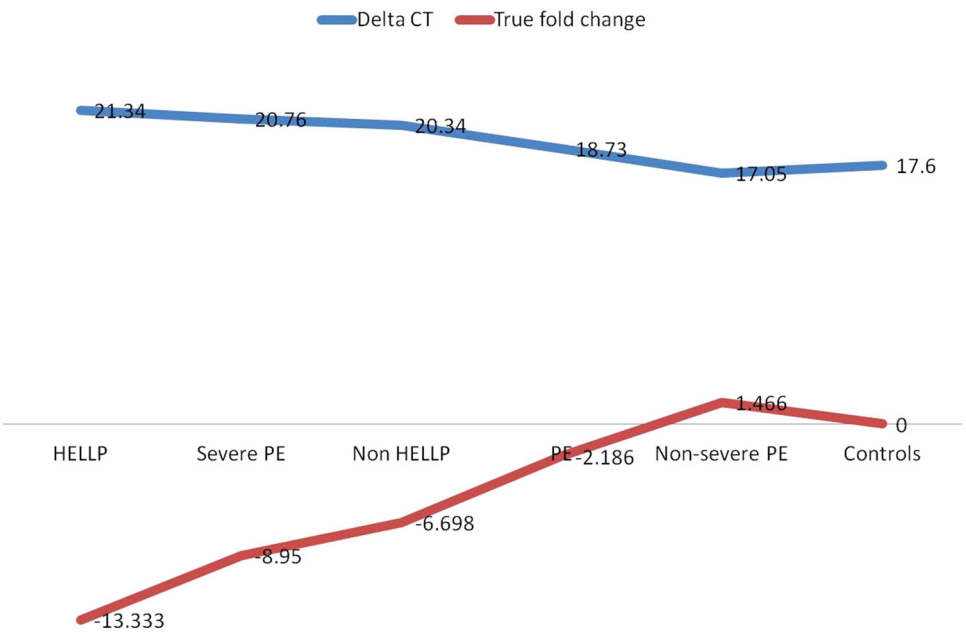

Fig. 2 Delta CT and true fold change of VEGF expression in PE and subgroups versus controls

finding of initial increase of VEGFA in non-severe PE and subsequent marked lowering in HELLP strengthens the existing hypothesis of decompensated VEGF being a major role player in PE. The exact pathophysiology of PE still remains investigational and requires further research.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Ethical Standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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