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Original Article

Immunohistochemical Expression of Placental Vitamin D Receptors in Pregnancies Complicated by Early and Late-Onset Preeclampsia

Dzenis Jelcic[a]*, Velibor Puzovic[c], Benjamin Benzon[b], Ivan Palada[d], Jelena Jerković[a,d], and Marko Vulic[a,b,d]

[a]*Department of Gynecology and Obstetrics, University Hospital of Split,*
[b]*University of Split School of Medicine,*
[d]*University Department of Health Studies of the University of Split, 21000 Split, Croatia,*
[c]*General Hospital Dubrovnik, Department of Pathology and Cytology, 20000 Dubrovnik, Croatia*

The aim of our study was to determine whether the immunohistochemical expression of placental vitamin D receptors is altered in pregnancies complicated by preeclampsia. Vitamin D receptor expression was immunohistochemically analysed in the placentas of three groups: a control group, and early- and late-onset preeclampsia groups. Total immunohistochemical intensity staining of placentas showed that the control group had a median vitamin D receptor (VDR) expression significantly higher than the placentas of mothers with early- and late-onset preeclampsia. There was no difference among the three groups in a semiquantitative analysis of VDR staining of the stroma only. Vitamin D receptors showed lower median expression in preeclampsia-affected pregnancies, especially early-onset preeclampsia. Therefore, Vitamin D receptor expression may be an important marker for normal placentation and preeclampsia onset.

Key words: vitamin D receptor, immunohistochemistry, early and late-onset preeclampsia

Preeclampsia is a serious complication of pregnancy that affects 2-7% of pregnancies and is associated with substantial maternal and fetal morbidity and mortality [1,2]. This pregnancy-induced disease is diagnosed by the combined presentation of new-onset hypertension and proteinuria, and it can progress to multi-organ dysfunction including liver involvement, renal insufficiency, neurological or hematological complications, uteroplacental dysfunction and intrauterine fetal growth restriction [3]. Preeclampsia is understood to originate in the placenta [1,4]. Preeclampsia is considered to have two stages: abnormal placentation and maternal syndrome. Ischemic placenta, after abnormal placentation, produces excess levels of the antiangiogenic factors and induces maternal endothelial dysfunction leading to preeclamptic signs and symptoms [5]. Today, a clear distinction is made between early and late clinical forms of preeclampsia. Early-onset preeclampsia is often called placental; it is rare and usually combined with placental insufficiency and intrauterine growth retardation. Late-onset preeclampsia is called "maternal" preeclampsia, it is much more common (80% of all preeclampsia cases) and the placenta is not affected; the most probable cause is the mother's genetic predisposition [6]. The management of preeclampsia involves stabilization of the mother and fetus, followed by delivery at an opportune time, often preterm. Delivery is the only effective treatment for preeclampsia, and delivery results in complete healing. However, today we know that preeclampsia, especially early-onset preeclampsia, poses a significant risk factor for cardiovascu-

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*Corresponding author. Phone: +385-994420044
E-mail: dzenis.jelcic@gmail.com (D. Jelcic)

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lar and metabolic diseases later in the mother's life [7].

The beginning of intrauterine life development requires an immune balance that tolerates the allogenic fetus and maintains reactivity against pathogens. Dysregulation of this balance mediates the pathogenesis of pregnancy-related disorders like preeclampsia. Vitamin D deficiency is known to increase the risk of preeclampsia [8]. The active form of vitamin D, or 1,25 dihydroxy vitamin D, binds to vitamin D receptors (VDRs) [9]. VDRs act as transcription factors whose products are involved in cellular proliferation, differentiation, invasion and apoptosis, and they play pivotal roles in calcium homeostasis and bone mineralization [10]. VDRs are strongly expressed in the decidua and placenta during gestation and participate in immunomodulation at the maternal-fetal interface [11]. Additionally, VDRs are considered regulators of immunity, innate and adaptive, by their regulation of cellular proliferation, differentiation and apoptosis [12,13]. Recent studies have discussed the potential role of vitamin D as a regulator of trophoblast invasion in early pregnancy and have suggested that it acts to promote implantation due to its role in inflammatory pathways and immune function [14]. Since VDRs actively participate in pathophysiological processes at the cellular level, it is possible that they play multiple roles related to placentation, angiogenesis, oxidative stress, and altered inflammatory and immune responses in the development of preeclampsia. Recent studies have shown that placental VDR expression is decreased in pregnancies complicated with idiopathic fetal growth restriction, but not in those complicated with preeclampsia [15,16].

The diagnosis of preeclampsia in one pregnancy raises questions about essential causes, be they genetic or incidental, and hence the recurrence risk for future pregnancies. Our research providing insights into the placental expression of VDR, an important hallmark of normal placental development, might help to elucidate these important questions. The aim of the present study was to determine whether the defective placentation in preeclampsia is associated with decreased vitamin D receptor expression in the placenta.

Materials and Methods

This study included pregnant women delivered in the General Hospital of Dubrovnik in Croatia in 2015-2016. The study was conducted in accordance with the Declaration of Helsinki. All procedures were approved by the local Ethical Committee Board (UR 0804/2015), and all procedures were carried out with the adequate understanding and written consent of each subject.

The inclusion criteria were all pregnant women whose pregnancy was terminated by Cesarean section or vaginal delivery due to early- or late-onset preeclampsia, as well as normal-term pregnancies without delivery complications (controls). The exclusion criteria were all pregnant women who suffered from other diseases, preterm labor, severe infection during pregnancy or women not willing to take part in this study. We assigned the women and their respective samples to three groups: normal gestation with term delivery, late-onset preeclampsia, and early-onset preeclampsia with intrauterine growth restriction (IUGR). We applied the mean difference formula in each group to determine the sample size, which was 10 samples for each group. The total number of subjects in this study was 61, with 24 subjects in the normal-gestation group, 17 subjects with late-onset preeclampsia, and 20 subjects with early-onset preeclampsia.

The subjects were pregnant women whose delivery dates were based on obstetric medical indications. We defined normal gestation as pregnancy until term without complications and based the definition of preeclampsia on the 2013 ACOG criteria, including blood pressure of $\geq$140/90 mmHg with proteinuria after 20 weeks of pregnancy in previously normotensive women. Early-onset preeclampsia was defined as preeclampsia diagnosed before 34 weeks, while late-onset preeclampsia was defined as preeclampsia diagnosed at or after 34 weeks of pregnancy. The major difference between early- and late-onset preeclampsia is that early-onset preeclampsia is usually complicated by IUGR and/or other organ involvement with severe clinical features. IUGR was defined as fetal weight lower than the 10th percentile on the growth chart. Patient characteristics are shown in Table 1.

After the subjects gave birth at the General Hospital of Dubrovnik, their placental tissues were taken to the Department of Pathology, where they were prepared for immunohistochemical staining for vitamin D receptors. Placental tissue samples were fixed in 10% buffered formalin and subjected to the standard procedure for routine pathohistological diagnosis. Two samples, 3:2 cm, of full thickness were taken from the central placenta and the part of the placenta between the center and the

Table 1

Variable/Group	Control group	Late-onset preeclampsia group	Early-onset preeclampsia group
Number	24	17	20
Parity(%)			
1	45	36	55
2	20	36	27
3	35	28	9
4	0	0	9
BMI before pregnancy (kg/m^2, median, IQR)	24.5 (23.5–25.1)	24.1 (23.05–25.13)	24.5 (23.83–25.58)
Term - weeks of gestation(%)			
Preterm (<37)	0	7	5
Early term (37 0/7–38 6/7)	29	29	75[a]
Full term (39 0/7–40 6/7)	53	57	15[a]
Late term (41 0/7– 41 6/7)	18	7	5
Mother age (years, average ± SD)	30.35 ± 5.35	29.86 ± 6.4	28.82 ± 7.3
Gender of the baby (%)			
male	47	64	45
female	53	36	55
Baby birth weight (g, average ± SD)	3,663 ± 398.5	3,329 ± 631.5	2,304 ± 269[b]

[a]$p = 0{,}0407$ for comparison with other groups (χ^2 test); [b]$p < 0{,}0001$ for comparison with other groups (*t*-test with Welch correction).

margin. The material part together with the area of the central cotyledons was routinely processed in a histokinet, then embedded in paraffin and cut with a microtome into slices that were 4-5 micrometers thick. Every 10th section was stained with the standard hematoxylin and eosin method. The sections were examined with a light microscope to determine possible pathohistological changes, and remaining sections were used for immunohistochemical staining. For immunohistochemical staining, the standard avidin-biotin immunoperoxidase method was performed using an automatic staining machine (Tech Mate, Dako, Denmark) with a vitamin D receptor-targeted antibody (rabbit polyclonal antibody ab3508, Abcam, UK) as recommended by the manufacturer.

We assessed 2 blocks of tissue per placenta, taken from the central and peripheral positions of the placenta. As it is known that placental infarction is one of the characteristic findings in early-onset PE, during the quantification procedure we omitted those areas from counting, and only areas without placental infarction were taken into account. We quantified staining of images with an Olympus BX1 microscope. Differences in intensity of staining can be expressed as percentages of stained cells or intensities of color in the stained cells. We devised an original semiquantitative index combining these measures with equal weight. Thus, intensity of staining was analyzed as 0, no staining; 1, weak staining; 2, moderate staining; or 3, strong staining, while the percentage of positive cells was represented by scores of 0, negative; 1, 1-10%; 2, 10-50%, or 3, >50% stained cells. Final indices numbers were obtained by multiplying the intensity (0,1,2,3) and percentage of positive cells (0,1,2,3) and are represented as immunohistochemical index (IH index).

Descriptive analysis of continuous or ordinal variables was presented by medians and interquartile ranges. In cases of normal distribution, data are presented as means and standard deviations. Dunn's test was used to compare continuous or ordinal variables between two independent groups, and the Kruskal-Wallis test was used to compare multiple independent groups. If data were normally distributed, a t test with or without Welch correction was used. In contrast to the continuous and ordinal variables, the χ^2 test was used for frequency data, and in the absence of the expected frequency, the Fisher exact test. We considered values of $p < 0.05$ statistically significant. The statistical software GraphPad Prism 7.0 (Graphpad, LaJolla, CA, USA) was used for analyses.

Results

The mean age of participants by group was 30.35 ± 5.35 years in the control group, 29.86 ± 6.4 years in the late-onset preeclampsia group, and 28.82 ± 7.3 years in the early-onset preeclampsia group. Parity was represented as "1" to designate the first childbirth, and 2, 3 and 4 to designate the second, third and fourth childbirth, *etc*. There was no statistically significant difference in parity among groups, but it seemed noteworthy that primigravidas (first-pregnancy women) comprised over half (55%) of the the early-onset preeclampsia group. There were no significant differences in anthropometric variables (BMI) between groups. There was no difference in gender of neonates among groups, but 64% of the neonates in the late-onset preeclampsia group were male. There was a statistically significant difference in the term of delivery, particularly in the early-preeclampsia group, of whom 75% had early-term deliveries and only 15% had full term deliveries ($p < 0.0407$). There was a statistically significant difference in the birth weight of neonates, with neonates of the early-onset preeclampsia group having significantly lower birth weights ($p < 0.0001$).

Hematoxylin and eosin staining. Hematoxylin and eosin staining was used to show morphological features of early-onset preeclampsia with the villous trees that displayed stromal fibrosis and fibrinoids (Fig. 1A). Late-onset preeclampsia displayed villi that were not completely formed and stromal channels with circulating macrophages present in the villi (Fig. 1B). In Fig. 1C, we also show a healthy control group placenta with normal morphology (Fig. 1C).

Immunohistochemistry. The immunohistochemical analysis of VDR showed weak to moderate expression in early- and late-onset preeclampsia, while in the control group VDR expression was strong when both stroma and trophoblasts were considered (Fig. 1D-F). When only the stroma was evaluated, VDR expression was weak in all groups; when only the trophoblast was evaluated, VDR expression was moderate in the control group and weak in early- and late-onset preeclampsia. Although placental infarction, the characteristic finding in early-onset PE, was observed in all early-onset PE samples, we excluded those areas from the VDR-positive cell counts.

Semiquantitative analysis of total immunohistochemical staining intensity of placentas (stroma and trophoblast) showed that placentas of the control group had a median VDR expression 0.5 points higher than

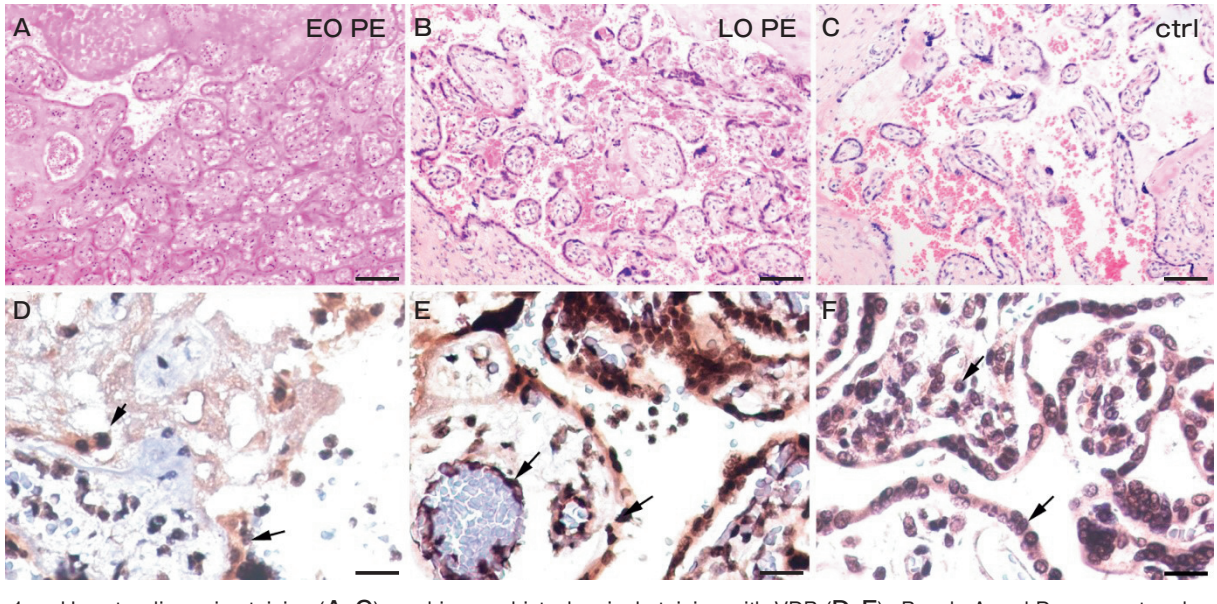

Fig. 1 Hematoxylin-eosin staining (**A–C**), and immunohistochemical staining with VDR (**D–F**). Panels A and D represent early-onset preeclampsia (EO PE); panel B and E represent late-onset preeclampsia (LO PE), and panel C and F represent healthy controls (ctrl). Representative VDR-positive cells are shown by arrows; all cells with brown cytoplasmic staining were considered VDR-positive. Scale bar from A–C panel is 100 μm, and from D–F is 25 μm.

the placentas of mothers with late-onset ($p = 0.0161$) and early-onset preeclampsia ($p = 0.0075$) (Fig. 2A). However, there was no difference in the semiquantitative analysis of stromas between the groups (Fig. 2B). Regarding the intensity of immunohistochemical staining of placentas, the trophoblasts in the late-onset ($p = 0.0195$) and early-onset preeclampsia group ($p = 0.0049$) had median VDR expressions 1 point less than the control group (Fig. 2C). In contrast to the semi-quantitative immunohistochemical staining intensity of the stroma, the analysis of the IH index found differences in the median VDR expression of 1 point between the control group and the early-onset preeclampsia group ($p = 0.0348$). Pregnant women who had late-onset preeclampsia also had a median VDR expression 1 point lower than in the control group, but the difference did not reach statistical significance ($p = 0.0725$) (Fig. 2D).

When the expression of VDR in the trophoblast was quantified by the IH index, the same trends could be observed as in the semiquantitative analysis, but without statistical significance (Fig. 2E). When the median IH index of both the stroma and trophoblast were compared among groups, the early-onset preeclampsia group had a lower IH index than the control group ($p = 0.0494$) (Fig. 2F).

Discussion

Preeclampsia is a well-researched pregnancy disorder with known risk factors such as advanced maternal age (> 35 years) [17, 18]. Other studies show increased risk for preeclampsia in very young pregnant women (18 to 24 years) as well as pregnant women of advanced age (40 to 54 years) [19]. Our study showed no difference in median maternal age among groups, which is in accordance with a study conducted a few years ago in Iran on a sample of more than six thousand women [20].

Bartsch *et al.* provided evidence from a meta-analyses that nulliparity is a risk factor for preeclampsia. Our results did not uphold this finding: in our study there was no statistically significant difference among groups in maternal parity although primiparous women did comprise 55% of the group with early preeclampsia [17]. However, our sample size was much smaller than in their study. Insufficient statistical power could also be why no significant difference was found among the

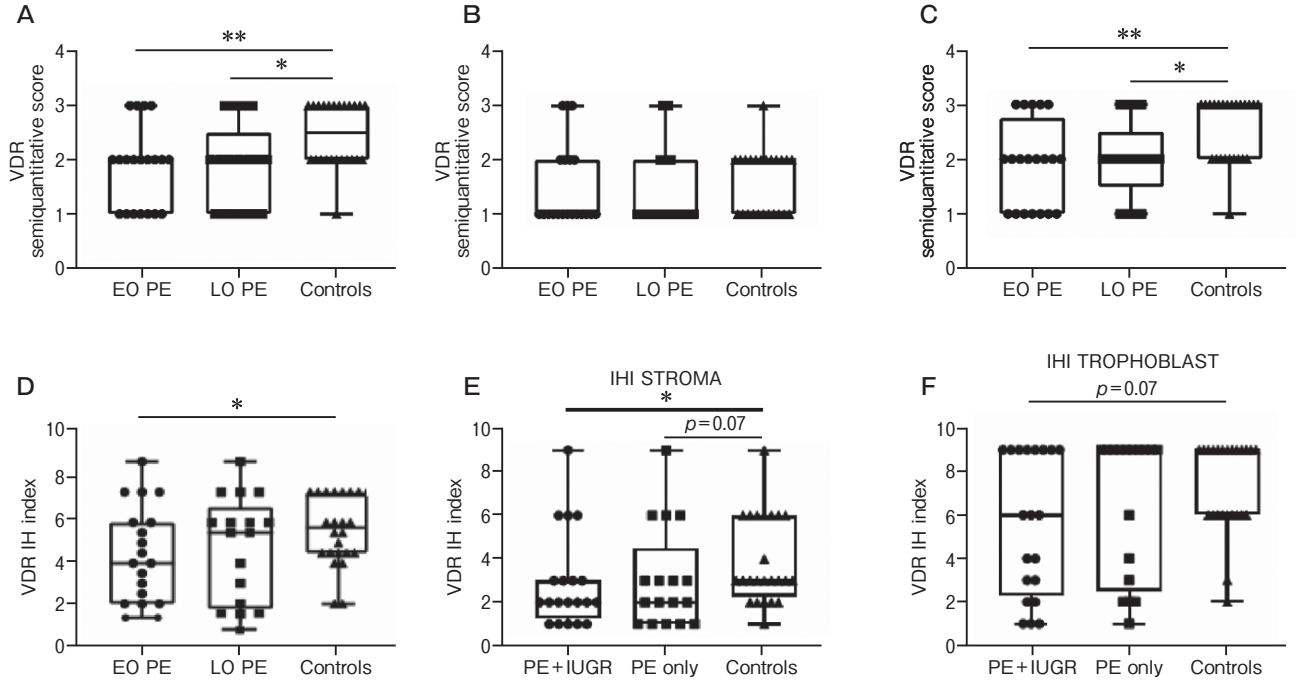

Fig. 2 Total staining of placental tissue (stroma and trophoblast) determined semiquantitatively (2A), stroma staining (2B), trophoblast staining (2C), immunohistochemical index of stroma and trophoblast (2D), immunohistochemical index of stroma (2E), immunohistochemical index of trophoblast (2F). $^{*}p < 0.05$ $^{**}p < 0.01$

groups in anthropometric variables, as in other studies with small numbers of samples [16].

In our study we found no significant difference in BMI among groups while Phipps *et al.* found that high BMI is a progressive risk for preeclampsia. Again, they had a much larger cohort than ours [21]. Our study also did not show a statistically significant difference in the sex of the newborns among the observed groups, although 64% of the newborns in the late-onset preeclampsia group were male. This trend is not in accordance with other, larger studies, for instance one in Sweden enrolling 1,158,276 births, which found that male newborns were associated with a significantly lower risk for maternal preeclampsia [22], or a study performed in Japan in 2001-2005 on 241, 292 pregnant women showing that female fetal gender is risk factor for preeclampsia [23].

Recent studies of NEMO, an essential regulator of nuclear factor-κ B (NF-κB) in cytoplasmic and nuclear cellular compartments, showed that reduction of NEMO protein in the preeclamptic placenta may activate one of the molecular pathways influencing the development of preeclampsia, especially in pregnancies with a female fetus [24].

Our research showed a statistically significant difference in terms of delivery particularly in the early-preeclampsia group, in whom 75% of deliveries occurred early and only 15% were full-term. Our study was limited by our definitions of the term pregnancy and preeclampsia because the vast majority of pregnancies with early-onset preeclampsia were completed before 37 weeks of gestation. As to the time of delivery, close monitoring is essential to optimize maternal and fetal outcomes. Preterm planned delivery in preeclampsia was been shown to reduce maternal morbidity but lead to more neonatal unit admissions for the infant due to prematurity. In a multicenter, randomized controlled trial done in 46 maternity units across England and Wales, Chappell *et al.* concluded that the timing of delivery in late-preterm (34th to 37th gestation week) preeclampsia is an important issue to discuss with pregnant women. In their study, early delivery reduced maternal morbidity and while it led to more admissions to neonatal intensive care units, they found are no indications of greater neonatal morbidity [25]. The HYPITAT trial, a multicenter randomised-controlled trial, concluded that induction of labor should be advised for women with even a mild hypertensive disease after 37 weeks of gestation because earlier delivery was associated with improved maternal outcome [26].

Our results showed a statistically significant difference in birth weight of neonates among the different groups, with neonates of the early-onset preeclampsia group having lower birth weights. The median birth weight of newborns was 3,663 ± 398.5 g in the control group, 3,329 ± 631.5 g in late-onset preeclampsia, and 2,304 ± 269 g in early-onset preeclampsia. Our findings support the new insights that early-onset preeclampsia starts from the beginning of pregnancy with defective placentation and results in intrauterine growth retardation for the same reason, while late-onset preeclampsia is connected with normal placentation and maternal genetic predisposition to cardiovascular and metabolic disease, usually appears after 34 weeks of gestation, and is not connected with intrauterine growth retardation [27].

In this study, semiquantitative analysis of total staining of placentas (stroma and trophoblast) showed that placentas of the control group had a significantly higher median VDR expression (0.5 points higher) than the placentas of mothers with early- or late-onset preeclampsia. This accords with a recent study of Hutabarat *et al.* which showed trends of lower mean values of VDR staining in early- and late-onset preeclampsia compared to normal pregnancies, but a significant difference only between the normal pregnancy and intrauterine growth restriction groups [15]. The findings of Nguyen *et al.* from 2015 are also concordant [16]. Our studies in VDR expression had a larger number of samples than that in the study by Hutabarat *et al.* [16]. Furthermore, in this study we calculated an immunohistochemical index (product of staining intensity and percentage of positive cells) in the stroma and trophoblast, and our results showed significantly lower median expression of VDR in the group with preeclampsia, especially in the early-onset preeclampsia group. Although a number of previous studies have found an association between maternal vitamin D deficiency and early-onset and severe preeclampsia, there have been no studies about VDR expression in placenta of preeclamptic women calculated in this way [28, 29].

The etiology of preeclampsia is still unclear, but today we know that defective placentation, probably the result of a combinations of factors that affect the trophoblast and decidua, is a reason for a culminant abnormal immune response and clinical symptoms of

early-onset preeclampsia [27]. Vitamin D has anti-inflammatory action and can downregulate pro-inflammatory cytokine expression. In addition, reduced expression of VDR as found in early-onset preeclampsia can disrupt metabolism and lead to deficits in the expression of numerous genes involved in cell proliferation and differentiation [30].

A systematic review and meta-analysis of randomized controlled trials from 2015 showed that while vitamin D supplementation during pregnancy is associated with increased circulating 25(OH) vitamin D levels, birth weight and birth length, it is not associated with other maternal and neonatal outcomes like preeclampsia [31]. Gallo *et al.* supported this result with their meta-analysis from 2020 [32]. Our findings did not clarify whether the changes associated with placental VDR expression in preeclampsia pregnancies are a cause or a consequence of the pathophysiological defects observed in the preeclampsia-affected placenta. New studies should investigate whether prenatal vitamin D supplementation can increase VDR expression in the placenta and reduce the risk of preeclampsia in high-risk women. Genetic studies on the predisposition to preeclampsia should also continue.

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