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The investigation of serum nectin-4 levels in patients with early onset preeclampsia

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ABSTRACT

Aims: We aimed to investigate the level and predictive value of soluble nectin-4 in early onset preeclampsia (EOPE).

Methods: Forty-three patients with EOPE and 41 healthy normotensive pregnant women participated in this prospective case-control study. The groups were matched for gestational age and gravidity. Serum nectin-4 levels were compared between groups. The ROC curve was drawn to show the predictive value of nectin-4 for EOPE. Patients were followed up until the end of labor, and perinatal outcomes were recorded.

Results: The demographic characteristics of the two groups were similar. Serum nectin-4 level was significantly increased in EOPE cases compared to controls (226.46±119.6 ng/ml vs. 156.54±44.8 ng/ml, p=0.001). The ROC showed that at > 160.938, the sensitivity and specificity were 67.44% and 82.93%, respectively [AUC:0.822, (CI:0.724 - 0.897), and (p< 0.001)]. Significant inverse correlations were found between nectin-4 levels and poor obstetric outcomes.

Conclusion: Maternal serum nectin-4 levels were significantly higher in patients with EOPE compared with controls. Increased nectin-4 levels may contribute to the development of EOPE through possible oxidative, immunological, and inflammatory mechanisms adversely affecting trophoblastic cells.

Keywords: Early preeclampsia, nectin-4, preeclampsia, pregnancy

INTRODUCTION

Preeclampsia (PE) is a progressive multisystem disorder of pregnancy associated with new-onset hypertension that typically occurs after the 20th week of pregnancy. It is caused by dysfunction of the placenta and maternal blood vessels and regresses in a variable time interval after labor. PE is classified by time of onset as early-onset PE (EOPE) before 34 weeks and late-onset PE (LOPE) after 34 weeks. Compared with LOPE, EOPE has a higher risk of adverse pregnancy outcomes due to moderately early, very early, and extremely early delivery.[1-3]

Preeclampsia is assumed to be a two-stage disease resulting from defective trophoblast invasion and failure of spiral arterial remodeling as the main step of pathogenesis.[4] The LOPE is more common (90%) and is accepted as a mild maternal reaction to pregnancy.[5] In EOPE (10%), unlike LOPE, due to the improper placental invasion, the diffuse placental ischemia and the resulting oxidative stress begin in early gestation. Therefore, EOPE can lead to severe disease resulting in perinatal and maternal morbidity and mortality.[6]

Numerous inflammatory molecules play a role in the etiopathogenesis of preeclampsia.[7,8] Increased TNFα expression in preeclampsia has been shown to contribute significantly to placental and endothelial damage.[9] The level of disintegrin and metalloprotease 17 (ADAM -17), which is responsible for the formation of soluble TNFα, has been observed to be increased in preeclamptic pregnancies. The ADAM -17 also takes role in the shedding of nectin-4 into systemic circulation. The nectins are calcium-independent immunoglobulin-like cell adhesion molecules and play a central role in cellular junctions, as well as physiological regulations.[10] The placenta expresses for types of nectins; nectin-1, nectin-2, nectin-3 and nectin-4. They are localized at tight junctions and gap junctions of syncitiotrophoblasts.[11] nectin-4 is

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a relatively novel member of the nectin family that has only been detected in placenta and airway epithelium in healthy subjects.[10,12] nectin-4 has been researched since it serves as a viral receptor, is associated with oral and facial malformations, and is currently defined as a marker and potential therapeutic target in several cancers.[13]

Nectin-4 is expressed on the apical cell membranes of syncytiotrophoblasts and, when overexpressed, can induce a cytotoxic effect of NK, similar to EOPE, leading to placental dysfunction. Because nectin-4 is located on the apical cell membranes of syncytiotrophoblasts, it is possible to detect it in the maternal circulation.[10-13] Therefore, we hypothesized that the nectin-4 level might be elevated in EOPE patients, which has not yet been investigated in EOPE.

METHODS

The study was carried out with the permission of University of Health Sciences, İstanbul Kanuni Sultan Süleyman Health Research Center, Clinical Researches Ethics Committee (Date: 13.05.2020, Decision No: KAEK/2020.05.13). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

This prospective case-control study was conducted in Istanbul Kanuni Sultan Süleyman Health Center, Department of Perinatology from May to December 2020. The study group composed of 84 patients who referred to perinatology clinics in this process. Informed written consent was obtained from each participant. The data concerning the obstetric and general health of the cases were also recorded (ClinicalTrials.gov Identifier: NCT05098691).

There were 43 patients with EOPE included in the study group, and the 41 normotensive, healthy and gestational age, and gravidity-matched pregnant women, with normal arterial blood pressure, without proteinuria were stated as the control group.

The study group included spontaneous, singleton pregnancies above the 24 weeks of gestations with positive fetal cardiac activity. Exclusion criteria were determined as multiple gestations, eclampsia, HELLP syndrome, chronic hypertension, hypothyroidism, known malignancy, diabetes mellitus, presence of fetal or maternal infection, clinical signs of chorioamnionitis (maternal fever, vaginal discharge, fetal tachycardia), hepatic or renal failure, collagen vascular disease, Placenta previa or pregnancies accompanied congenital fetal abnormalities or aneuploidies. We also excluded patients with systemic diseases, history of preeclampsia, and patients who became pregnant by in vitro fertilization, which could alter the level of nectin-4 in the maternal circulation. In addition, women who were treated with magnesium sulfate, corticosteroids/non-steroidal anti-inflammatory, and illegal drug users were also excluded from the study.

Preeclampsia definition was performed according to the ACOG guidelines(1). After the 20th gestational week,the pregnant women with the systolic blood pressure (BP) ≥ 140/90 mmHg or the diastolic BP ≥ 90 mmHg in least two measurements taken four hours apart with the previous history of normal BP and the presence of proteinuria (≥300 mg/24 h urine collection, or protein/creatinine ratio of ≥0.3 mg/dl, or a dipstick reading of 2+ protein) were diagnosed as preeclampsia.

We were taken blood samples of the cases, during the preeclampsia diagnosis for the study group and during routine antenatal follow up before labor for the control group. The two groups were matched in terms of gestational ages at the time of the maternal blood sample collection. Maternal age, body mass index (BMI), smoking status, gravidity, gestational week, amount of proteinuria, systolic and diastolic BP levels, and serum nectin-4 concentrations were recorded. A calibrated sphygmomanometer was used for the measurement of BP.

Venous blood sampling was performed after one night of fasting for all participants at the time of initial diagnosis. The blood samples were centrifuged at 4000 rpm for 10 minutes at room temperature. The obtained serum samples were frozen immediately and stored at -80°C up to the time of serum analysis. Nectin-4/human poliovirus receptor related protein (PVRL4) levels in the samples were measured using a double-antibody sandwich ELISA (Wuhan USCN Business Co., Ltd). The microtiter plate provided in this kit has been pre-coated with an antibody specific to Poliovirus Receptor Related Protein 4 (PVRL4). Standards or samples are then added to the appropriate microtiter plate wells with a biotin conjugated antibody specific to Poliovirus Receptor Related Protein 4 (PVRL4). Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain Poliovirus Receptor Related Protein 4 (PVRL4), biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm±10nm. The concentration of Poliovirus Receptor Related Protein 4 (PVRL4) in the samples is then determined by comparing the O.D. of the samples to the standard curve. All laboratory measurements were performed simultaneously in the same laboratory by the same technician.

Statistical Analysis

All statistical analyses were performed using the RStudio integrated development environment for statistical computing (Affero General Public License v3; published 2011. RStudio for Linux, version v2021.09. 4+403.pro3 Ghost Orchid; September 19, 2022; developed by Posit, PBC.) to analyze the data. Variables were examined using visual (histogram, probability plots) and analytic methods (Kolmogrov-Simirnov/Shapiro-Wilk's test) to determine if they were normally distributed. Levene's test was used to assess the homogeneity of variance. For the non-parametric values, descriptive analyses were presented using medians and quartiles. Mann-Whitney U tests were performed to compare the nonnormally distributed numerical data between groups. For the categorical variables, descriptive analyses were presented using frequency and percentage. Relationships between categorical variables were analyzed using the chi-square test or Fisher's exact test (when the assumptions of the chi-square test assumptions do not apply because of low expected cell counts). The predictive capacity of nectin-4 levels for EOPE was analyzed using ROC (Receiver Operating Characteristics) curve analysis, and the sensitivity, specificity, AUC (Area Under Curve) value, positive likelihood ratio, and negative likelihood ratio were presented. When examining the relationships between non-normally distributed and/or ordinal variables, the correlation coefficients and their significance were calculated using the Spearman test. An overall 5% type- I error level was used to infer statistical significance. A p-value of less than 0.05 was considered a statistically significant result.

Power analysis was performed using G-power software (G-power v3.1.9.2, Kiel College, Kiel, Germany). The difference between two independent means showed that the study reached a power of 0.94.

RESULTS

A total of 84 patients were included in the study. Forty-three of them were diagnosed with EOPE, and 41 were control subjects. There were no statistically significant differences between groups in demographic characteristics such as maternal age, gravidity, body mass index (BMI), and smoking status ($p > 0.05$). The gestational weeks of the groups at maternal serum collection were similar ($p=0.37$). Systolic and diastolic blood pressure were higher in the EOPE group at gestational week at blood sampling ($p< 0.001$). Compared to the control group, serum levels of nectin-4 were significantly higher in the EO preeclampsia group (156.54±44.8 vs. 226.46±119.6, $p=0.001$) (Table 1).

Table 1. The demographic characteristics, amount of proteinuria and serum nectin-4 levels of the cases

	Early-onset preeclampsia group (n=43)	Control group (n=41)	p
Age (years)	26 (21-32)	27 (21-34)	0.110
Gravidity	2 (1-2)	2 (1-3)	0.260
BMI (kg/m²)	28 (25-32)	27 (24-31)	0.370
Gestational age at serum sampling (week)	31 (28-32)	31(29-32)	0.370
Smoking status +/-n (%)	9/34 (29.9%-79.1%)	7/34 (17.1%-82.9%)	0.863
Systolic blood Pressure (mm/Hg)	153 (145-176)	121 (110-128)	<0.001
Diastolic blood pressure (mm/Hg)	97 (88-126)	72 (62-81)	<0.001
Proteinuria (mg/24 hours)	3782 (1161-5247)	N/A	N/A
Nectin-4 levels (ng/ml)	190.173 (141.728-244)	124.79 (100.58-153.247)	0.001

BMI, body mass index; kg/m², milligrams per square meter; mmHg, millimeter of mercur; mg, milligram; ng/ml,nanogram per milliliter.Data are expressed as median (Q1-Q3), or frequency (percentage) where appropriate. A p value of <0.05 indicates a significant difference. Statistically significant p-values are in bold.

Compared with the control group, gestational age at delivery was lower in the EOPE group, resulting in lower birth weight and a higher rate of adverse fetal outcomes, including low APGAR scores at the first and fifth minutes and a higher rate of admissions and longer duration of treatment in the neonatal intensive care unit ($p<0.05$) (Table 2).

Table 2. Peripartum outcomes of the groups

	Early-onset preeclampsia group (n=43)	Control group(n=41)	p
Birth weight (gr)	1681 (1156-1865)	3212 (2818-3513)	<0.001
Birth length (cm)	41 (35-44)	46 (44-49)	<0.001
APGAR 1st min.	5 (3-8)	7 (6-9)	<0.001
APGAR 1st min <7	23/20 (53.5%/46.5%)	6/35 (14.6%/85.4%)	<0.001
APGAR 5th min.	7 (5-9)	9 (8-9)	<0.001
APGAR 5th min<7	12/31 (27.9%/72.1%)	1/40 (2.4%/97.6%)	0.003
Gestational age at delivery (weeks)	31 (28-33)	37 (35-39)	<0.001
Gender of babies (male/female)	25/18 (58.1%-41.9%)	21/20 (51.2%-48.8%)	0.676
Mode of delivery (vaginal/ceserean)	5/38(11.6%/88.4%)	25/16(61%/39%)	<0.001
Fetal distress emergency ceserean	10 (23.2%)	2(4.8%)	0.036
NICU admission	23/20(53.5%/46.5%)	10/31(24.4%/75.6%)	0.012
NICU duration(day)	17 (10-32)	0 (0-5)	<0.001

gr, gram; cm, centimetre; min, minute; NICU, neonatal intensive care unit. Data are expressed as median (Q1-Q3), or frequency (percentage) where appropriate. A p value of <0.05 indicates a significant difference. Statistically significant p-values are in bold.

The diagnostic value of nectin-4 was tested by ROC analysis. The ROC analysis showed that at > 160.938, the sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio were 67.44%, 82.93%, 3.95, and 0.39 respectively [AUC:0.822, (CI:0.724 - 0.897), and (p< 0.001)] (Figure 1).

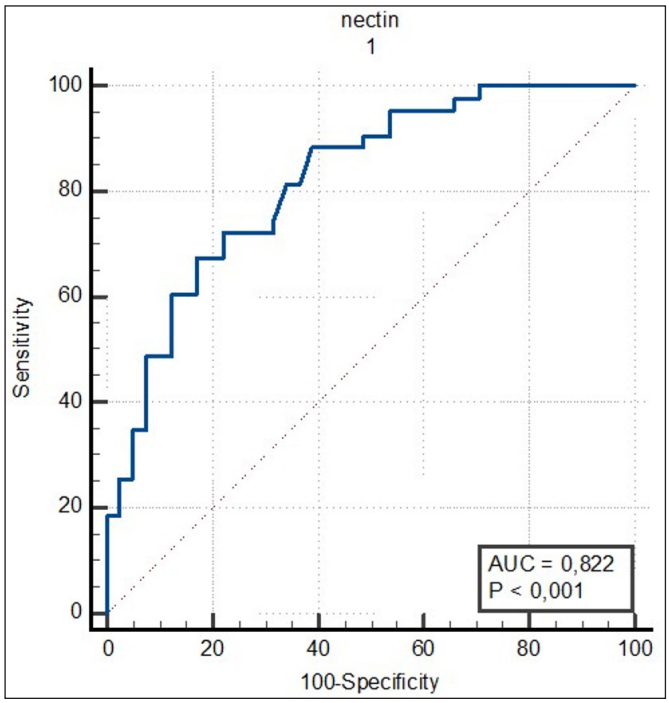

Figure 1. Diagnostic value of nectin-4

Significant inverse correlations were found between nectin-4 levels and poor obstetric outcomes. Also, there was a positive correlation between serum nectin-4 levels and the amount of proteinuria (r=0.58, p<0.001)(Table 3).

Table 3. The correlations between serum nectin-4 levels and intrapartum outcomes

Variable	Nectin-4 levels n	r	p-value
Birthweight	84	-0.27	0.010
Gestational week at birth	84	-0.30	0.005
1st min APGAR score	84	-0.42	<0.001
5th min APGAR score	84	-0.32	0.003
Fetal distress	84	0.58	<0.001
Diyastolic blood pressure	84	0.25	0.020
Proteinuria	43	0.58	<0.001

min, minute.

DISCUSSION

Preeclampsia is a multisystem disorder and one of the most important causes of perinatal morbidity and mortality, especially while the condition is of early onset. Despite the accumulating evidence indicating the underlying cause as the placenta, the etiology of the disease is still elusive. The abnormally shallow trophoblast invasion indicates the possibility of altered expression of adhesion molecules in preeclampsia.[14] Nectins are adhesion molecules which were also shown to be expressed on cellular membranes of trophoblast.[8] In this study, nectin-4 was found to be significantly increased in EOPE compared to gestational week matched healthypregnant women.

Early and late preeclampsia have been assumed as the diseases with distinct characteristics. EOPE is commonly associated with placental dysfunction, low placental volume, abnormal Doppler findings, fetal growth restriction, multiorgan dysfunction, and adverse maternal and neonatal outcomes, whereas late-onset preeclampsia appears rather as a maternal disorder.[4-6] The factors responsible from the pathogenesis of preeclampsia includes abnormal placentation, immunological mechanisms as a key point, and oxidative distress. Abnormal placentation was indicated as the start point of EOPE with a genetic tendency leading to high recurrence risk and running across the generations within same families.[5,15] The level of immunological factors were also reported as more altered in EOPE compared to LOPE.[16] Since the nectin-4 was reported to present on trophoblastic cells, we investigated whether nectin-4 level is related with EOPE or not.

In normal placentation, extravillous trophoblasts are responsible for the deep infiltration of uterine wall and invading the muscular walls of the uterine arteries and endovascular trophoblasts replaces the endothelial cells. This process increases the blood flow to the intervillous space by altering the vascular conductance. In EOPE, impaired growth of the villi has been attributed to the inadequate formation of the cytotrophoblastic shell early in pregnancy. Reduced capacity of trophoblasts to invade the myometrial part of spiral arteries lead to restricted blood supply through the uteroplacental circulation and following hypoxia.[6] The maternal adaptation to fetal antigens includes the maternal tolerization to paternal antigens in seminal plasma at the preconceptual phase is crucial for normal placentation. After conception, recognition of fetal MHC class 1 molecules on trophoblasts as self by regulatory T cells and NK cells allows placentation.[17] The villoustrophoblasts exposed to maternal blood are lack of MHC class I.However, the extravillous trophoblasts which invade the uterus was shown to have a nonclassical MHC class I repertoire (HLA-E and HLA-G in addition to HLA-C) which interact with NK cells and T cell receptors for immune tolerance. Complete failure in immune tolerance results in miscarriage, whereas the partial failure may result in preeclampsia. Besides the invasion process, the systemic immune response was also shown to be responsible for the pathogenesis of preeclampsia.[18] The shedding of the soluble and bound HLA-G isoforms into systemic circulation was

shown to trigger immunomodulatory activities of NK cells and T lymphocytes in pregnancy. The soluble isoforms of HLA-G were reported in decreased levels in preeclampsia, even in the first trimester before the onset of the disease, indicating the disturbed immune modulation.[19-21] Both the impaired trophoblast invasion and systemic inflammatory response provide the basis for hypoxic environment.6

Nectins are adhesion molecules belonging to immunoglobulin superfamily and exhibit structurally related three immunoglobulin-like (V, C, C) domains in their extracellular side. Unlike the other nectins, nectin-4 is mainly expressed during embryogenesis and is only detected in trophoblasts and slightly in trachea in healthy human.[10,12] Ito et al.[8] examined the placental tissues of pre-eclamptic pregnant women (n=20) and uncomplicated pregnancies (n=20) and reported elevated nectin-4 expression in preeclampsia. They reported that trophoblastic cell migration was not impaired by the overexpression of nectin-4. However, the trophoblasts overexpressing nectin-4 were more vulnerable to the NK cell cytotoxicity and the cytotoxic attack by natural killer cells was significantly increased against nectin-4 overexpressing cells.[8] The mean gestational age was similar between the preeclamptic and normal group, on the other hand, the preeclampsia cases were not defined as early- or late-onset and only the severe preeclamptic patients were included. To the best of our knowledge, the current study is the first one to evaluate the maternal serum level of nectin-4 in EOPE.

The previous studies conducted on nectin-4 were generally concentrated on its significance in tumoral activity and poor prognosis in mainly bladder, lung, pancreas, and breast cancers. It was denoted that nectin-4 binds only the inhibitory receptor TIGIT (T-cell immunoreceptor with Ig and ITIM domains). By this way, theincreasing nectin-4 expression was reported to inhibit the NK cell activity more profoundly and decrease the NK response to cancer cells. The studies were also targeted nectin-4 and TIGIT in cancer immunotherapy.[22,23] TIGIT was also shown as a target to be promoted to achieve immune tolerance in repeating miscarriages in the study conducted by Fu et al.[24] The studies on immunomodulatory mechanisms of pregnancy revealed that decidual immune cells of maternal-fetal interface expressing TIGIT, which is a co-inhibitory receptor, triggers immunological tolerance. TIGIT was reported to inhibit NK cells' effector function and suppress their dendritic cell costimulatory ability. The expression of TIGIT in decidual CD4+ T cells and NK cells at the transcriptional level was also shown to be upregulated by progesterone.[24,25] Even though NK cell activity was not evaluated in our study, the literature supports the evidence on increased NK cell activity EOPE. The conflicting data reported by Ito et al.[8] on increased NK cell activity despite the increased nectin-4 expression of trophoblastic cells, however, TIGIT receptivity was not studied. On the other hand, in a recent study, Meggyes et al.[18] investigated the immune checkpoint receptors in EOPE, and they revealed that a subgroup of cytotoxic T lymphocytes had significantly lower levels ofTIGIT in EOPE compared to healthy controls.[19] In this regard, there may bemore complicated mechanisms in the etiology of preeclampsia which may lead to improper functioning of NK cells, TIGIT receptors and in which nectin-4 may be involved.

The inflammatory process and decreased uteroplacental perfusion in EOPE result with hypoxia and oxidative distress. The key inflammatory mediator released from NK and T cells is TNFα.[6,26] TNFα was found to be increased in preeclampsia, even in the early pregnancy, before the onset of the preeclampsia and emphasized as a candidate for predicting preeclampsia.[27] TNFα is released into systemic circulation in the soluble form by shedding via TNFα-converting enzyme, which is identical with ADAM17 (a disintegrin and metalloprotease 17). The expression of protease and sheddase ADAM17 was shown to be induced under hypoxic conditions.[26] ADAM10 and ADAM17 was shown to be increased in preeclamptic pregnancies and ADAM17 was also shown to be responsible from the increase in TNFα in preeclampsia.[28] The soluble nectin-4 is the extracellular domain of nectin-4 that is also released through the shedding into maternal circulation by the proteolytic activity of proteases ADAM-10 and 17.[12,27,29] Through the insight of the literature, the higher soluble nectin-4 levels detected in this study may be a consequence of the hypoxic environment and appears to be consistent with the mechanisms reported in the previous studies carried out on preeclampsia.

The role of angiogenesis for the normal placentation process was thought to support the idea that the disturbances in angiogenesis take role in etiopathogenesis of EOPE.[6] nectin-4 was reported to promote angiogenesis pathways in breast cancer in a recent study conducted by Siddhart et al.[30] They reported that upregulated ADAM-17 leading to shedding of the soluble nectin-4 ecto-domain which subsequently interacts with integrin-β4, the endothelial receptor for laminin taking role in cell adhesion, and promoting angiogenesis via Src, PI3K, AKT and iNOS. Our current study demonstrated increased levels of soluble nectin-4 in preeclampsia, even though, the literature denotes increased antiangiogenic factors and decreased angiogenetic factors as responsible forpreeclampsia

pathogenesis.[30,31] VEGF (vascular endothelial growth factor) is important in the stabilization of endothelial cells in in blood vessels. VEGF and placental growth factor (P1GF)-a member of VEGF family- also contribute to normal proliferation and implantation of trophoblastic cells. In previous studies, VEGF and P1Gf was shown to be decreased in preeclampsia as the main angiogenetic molecules, whereas the antiangiogenic soluble Fms-like tyrosine kinase-1 (sflt1) and soluble endoglin (sEng) increase.[31,32] On the other hand, thedecrease inthe VEGF level in preeclampsia was shown to be the result of increased expression of soluble Fms-like tyrosine kinase-1 (sFlt1) which bind and antagonize the VEGF. Sflt1 and soluble endoglin (sEng) which have been shown to be released by proteolytic activity of ADAM10 and ADAM17, respectively, were reported to increase in preeclampsia.[33,34] As we mentioned before, ADAM 10 and ADAM17 increases in hypoxia, resulting in an increase in soluble nectin-4 levels. In this regard, the aforementioned mechanisms appear to be consistent to support our findingsasincreasedsoluble nectin-4 levelsin EOPE.

It has not been determined at which gestational week the nectin-4 level has the highest diagnostic value for early preeclampsia. However, the studies that examined nectin-4 levels in preeclampsia cases after 24 weeks' gestation found statistically significantly higher nectin-4 levels in preeclampsia cases than in controls, which is consistent with the current study.[35]

Consequently, nectin-4 is a potential novel diagnostic biomarker for EOPE. It could be a valuable biomarker in complicated cases where differential diagnosis is required, such as chronic hypertension and proteinuria, SLE, etc.

Study Limitations

First, the serum samples for nectin-4 were taken after EOPE had developed. It would have been more informative if the serum samples had been taken before EOPE developed, which might indicate whether nectin-4 plays a role in the pathophysiology of EOPE and could be used in predicting EOPE.

CONCLUSION

Our results show that maternal serum nectin-4 level was significantly higher in EOPE than in controls and has diagnostic value for EOPE. A significant inverse association was found between nectin 4 levels and poor obstetric outcomes. It is possible that elevated serum nectin-4 levels contribute to the development of EOPE through oxidative, immunologic, and inflammatory mechanisms acting on trophoblast cells via receptive processes at the molecular level.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of University of Health Sciences, İstanbul Kanuni Sultan Süleyman Health Research Center, Clinical Researches Ethics Committee (Date: 13.05.2020, Decision No: KAEK/2020.05.13).

Informed consent: Written informed consent was obtained from all participants who participated in this study.

Referee Evaluation Process: Externally peer reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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