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Placental and serum levels of human Klotho in severe preeclampsia: A potential sensitive biomarker

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ABSTRACT

Introduction: The Klotho (KL) gene, initially defined as an anti-aging gene in mice, shares 86% of the amino acid sequence withthe human KL protein. The KL gene plays roles in endothelial nitric oxide production, angiogenesis, antioxidant enzyme production and protecting against endothelial dysfunction, all of which may be associated with preeclampsia (PE). Human KL is the precursor of the gene products: α-KL and β-KL. In this study, we evaluated the gene expression, serum and placental levels of human KL in women with severe PE, pregnant women with chronic hypertension and healthy pregnant controls. Also, the gene expression, serum and placental levels of human decorin (DCN) were evaluated.

Methods: A total of 36 patients with severe PE, 10 with chronic hypertension, and 28 with healthy controls were enrolled. Placental and serum levels together with of KL and DCN were measured by ELISA and alsogene expression of these were evaluated.

Results: Placental and serum KL levels were significantly higher in the PE than in the controls and in women with chronic hypertension. Serum DCN levels were significantly higher in the PE women compared to controls and pregnant women with chronic hypertension. Placental DCN was similar in PE and healthy controls. There was no significant difference in the gene expression of KL and DCN in the groups. The best cut-off level for human KL to identify the presence of PE was calculated as 12.48 pg/ml with a sensitivity of 100% and and specificity of 96%, whereas for DCN 62.33 ng/ml to assess the presence of PE with a sensitivity of 86.1% and a specificity of 88%.

Conclusion: Human KL may be a valuable marker for PE, with high sensitivity and specificity. It also appears to be more sensitive and specific than human DCN.

1. Introduction

Preeclampsia (PE) is the leading cause of maternal and perinatal morbidity worldwide [1]. Several pathophysiologic mechanisms, such as abnormal placentation, oxidative stress, placental ischemia and endothelial dysfunction, have been blamed as causes of PE [2–6]. Although the exact cause of PE is still being debated, it is clear that the placenta plays a central role by producing bioactive factors that act both locally and in the maternal compartment. Poor invasion and oxidative damage are the main pathologic mechanisms that have been suggested as the cause of placental damage.

Oxidative damage in the placenta gives rise to the characteristic placental histology defined as accelerated villous maturation. The association between prolonged oxidative damage and PE has been previously reported [7,8].

The Klotho (KL) gene was initially defined as an anti-aging gene in mice. It encodes a type I single transmembrane protein that shares 86% of the amino acid sequence with the human KL protein [9]. Studies on mice have shown that KL is associated with an extended life span and increased resistance to insulin and oxidative stress in vivo and in vitro [10]. KL is produced in the kidney and brain and to a lesser degree, in the placental syncytiotrophoblast [11,12]. In humans, KL has been found as an etiologic factor for cardiovascular diseases [13]. The KL gene plays a role in endothelial nitric oxide production, angiogenesis, antioxidant enzyme production and protecting against endothelial dysfunction, all of which may be associated with PE [14–16].

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Placental KL gene expression has been found to be decreased in the patients with PE [18]. However, in another study that measured soluble KL in maternal serum and placenta, the soluble KL in maternal serum was found to be higher in PE patients than in controls [19].

Decorin (DCN) is a protein that is encoded by chromosome 12q22. It has been reported that DCN limits the invasion and endovascular differentiation of extravillous trophoblast cells during early placentation by binding to vascular endothelial growth factor (VEGF) receptor-2. It also inhibits angiogenesis by depressing VEGF activity on endothelial cells [20]. It has been shown that DCN increases in maternal blood and placental tissue in preeclamptic patients [21].

In this study, we investigated the serum and placental levels of human KL and DCN in preeclamptic, chronic hypertensive and healthy pregnant patients. We also measured the mRNA expression of the KL and DCN genes in the maternal blood of the same patients.

2. Materials and methods

This cross-sectional study was conducted in the Department of Perinatology of theMedical Faculty at Trakya University between November 2017 and November 2018. Consecutive patients between 18 and 42 years of age with singleton pregnancies between 23 and 40 weeks of pregnancy who met the inclusion criteria were included in the study. The study sample consisted of three groups. The groups were pregnant patients with severe PE (Group I), normotensive healthy pregnant controls (GroupII) and chronic hypertensive pregnant patients (Group III). All subjects with chronic hypertension or severe PE met the criteria based on International Society for the Study of Hypertension in Pregnancy and The Society of Obstetricians and Gynaecologists of Canada diagnostic criteria for the disease [22,23]. Subjects with any form of preexisting hypertension or proteinuria were excluded from the study. Women with multiple gestations, renal disease, cardiovascular disease, HELLP syndrome and who smoked heavily and fetuses affected by chromosomal and/or congenital anomalies were also excluded. Also, women who delivered preterm due to clinically or laboratory confirmed chorioamnionitis or sepsis in the neonate were not included.

Gestational age determination was made by using the date of the last menstrual period and first trimester crown-rump length measurement. Estimated fetal weight below the 10th percentile was defined as intrauterine growth retardation (IUGR) [24].

All patients were monitored by our institute's protocol and gave birth according to clinical conditions. At the beginning, 83 patents were included in the study. Of these, 9 patients were excluded because of a chronic disease or delivery at another hospital. Finally, 74 pregnant women, including 28 with an uneventful pregnancy course and outcome, 36 with severe PE and 10 with chronic hypertensive pregnancy, were enrolled after being informed of the purpose and procedures of this study. All participants signed an informed consent form. The study protocol was approved by the ethical committee of Trakya University. The study was funded by the Scientific Research Projects of the Trakya University Medical Faculty (TUBAP 2017–208).

2.1. Placental sampling and analysis

All placentas were collected from the patients immediately after cesarean section or vaginal delivery. Fresh placentas were examined macroscopically and a placental segment that has no signs of necrosis, or inflammation was obtained from the central region of the placenta. The tissue was excised by using a sterile lancet. After the excision of the sample, the remaining placenta was sent for pathologic investigation.

The placental tissue samples were immediately gathered in sterile tubes and placed at −80 °C for storage until the procedure. On the day of the study, the placental tissues were taken from the freezer and approximately 200 mg of each placental tissue sample was homogenized in 1 ml of physiological saline solution. The tissue homogenates were centrifuged at 5000 × *g* for 5 min. The supernatants of the tissue homogenates were taken for the analysis. Then the KL and DCN levels in the samples were measured by ELISA method.

10 μLof sample tissue and 40 μL of standard solution were incubated together at 37 °C′ and washed. 50 μL stop solution was added to the solution and the result was read by MultiskanTM GO Microplate Spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts, USA).

Table 1
Demographic and clinical characteristics of the groups [Median (min.-max.)].

	Groups			p
	Preeclampsia (n = 36)	Controls (n = 28)	ChronicHypertension (n = 10)	
Age (years)	28 (18–40)	28.5 (18–41)	31.5 (20–42)	[a]0.239
Height (cm)	160 (149–182)	160 (145–177)	159.5 (154–173)	[a]0.909
Weight (kg)	78.5 (52–116)	82 (55–114)	100 (79–140)	[a]0.007
BMI	31.6 (21.1–42.9)	31.7 (20.2–41.8)	37 (28–56.8)	[a]0.01

BMI: Body mass index.
[a] Kruskal Wallis Test.

2.2. Biochemical measurements

Maternal blood collection was made at admission and after the diagnosis of PE or chronic hypertension was available. None of the patients were in active labour. Blood samples were collected from the pregnant women into tubes containing EDTA. The samples were centrifuged and stored at −80 °C. The concentrations of KL and DCN were measured using commercially available human KL (KTE61910; Abbkine Scientific Co., CA USA) and human DCN (KTE62128; Abbkine Scientific Co., California, USA) ELISA kits.

The respective interassay and intra-assay coefficients were < 11% and < 9% for Human KL and Human DCN values. The detection range for Human KL was between 10 U/L and 160 U/L. All samples were assessed in triplicate. To standardize the values all measured as U/L, they were converted to pg/ml, according to the manufacturer's guideline suggestions for a data comparison.

2.3. Gene expression

Following maternal venous blood collection, 2 ml fresh whole blood samples were collected into tubes containing EDTA for Total RNA extraction. Total RNA was extracted from whole blood samples using QIA amp RNA Blood Mini Kit according to the manufacturer's protocol. (QIAGEN, Germany).

After measuring the purity and concentration of RNA molecules using Nanodrop 2000C UV–Vis Spectrophotometer (Thermo Scientific, USA),normalization was performed to 250 ng in 10 μl of RNA sample. After normalization, 10 μl RNA was taken and added to the PCR solution. The RNA molecules were converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Invitrogen Thermo Fisher, Waltham, Massachusetts, USA). The cDNA samples were stored at −80 °C. On the day of the study, the samples were taken and the gene expression of KL and DCN were studied using a gene expression assay kit (TaqMan Gene Expression; Paisley, Scotland, United Kingdom). All samples were assessed in triplicate. Relativeexpression levels were calculated with ΔΔCt method for each sample after normalization against the housekeeping gene beta-actin as internal control.

3. Statistical analysis

Statistical analysis was performed using the NCSS 2007 (Version: 07.1.21) (NCSS; Kaysville, Utah, USA) program. The data were analyzed using descriptive statistical procedures (mean, median, frequency, standard deviation, minimum and maximum). A Shapiro-Wilk test was used to examine the continuous variables with normal and

Table 2
Obstetric characteristics of the patients.

		Groups			p
		Preeclampsia (n = 36)	Controls (n = 28)	ChronicHypertension (n = 10)	
Gravidity [n, (%)]	1	18 (50)	8 (28.6)	4 (40)	
	2	11 (30.6)	5 (17.9)	2 (20)	
	3	6 (16.7)	7 (25.0)	3 (30)	
	≥4	1 (2.8)	8 (28.6)	1 (10)	
	Median (min-max)	1.5 (1–6)	3 (1–7)	2 (1–4)	[a]0.023
Parity [n, (%)]	0	15 (41.7)	9 (32.1)	5 (50)	
	1	15 (41.7)	8 (28.6)	3 (30)	
	2	4 (11.1)	6 (21.4)	2 (20)	
	≥3	2 (5.6)	5 (17.9)	0 (0)	
	Median (min-max)	1 (0–3)	1 (0–6)	0.5 (0–2)	[a]0.2
Previous miscarriages [n, (%)]	0	34 (94.4)	21 (75.0)	6 (60)	[b]0.03
	1	1 (2.8)	5 (17.9)	3 (30)	
	≥2	1 (2.8)	2 (7.1)	1 (10)	
Gestational weeks at delivery	Median (Min-Max)	35 (28–38)	37 (26–41)	36 (32–39)	[a]0.038
IUGR	No	18 (50)	25 (89.3)	8 (80)	[b]0.002
	Yes	18 (50)	3 (10.7)	2 (20)	

IUGR: Intrauterine growth retardation.
[a] Kruskal Wallis.
[b] Pearson Chi-Square.

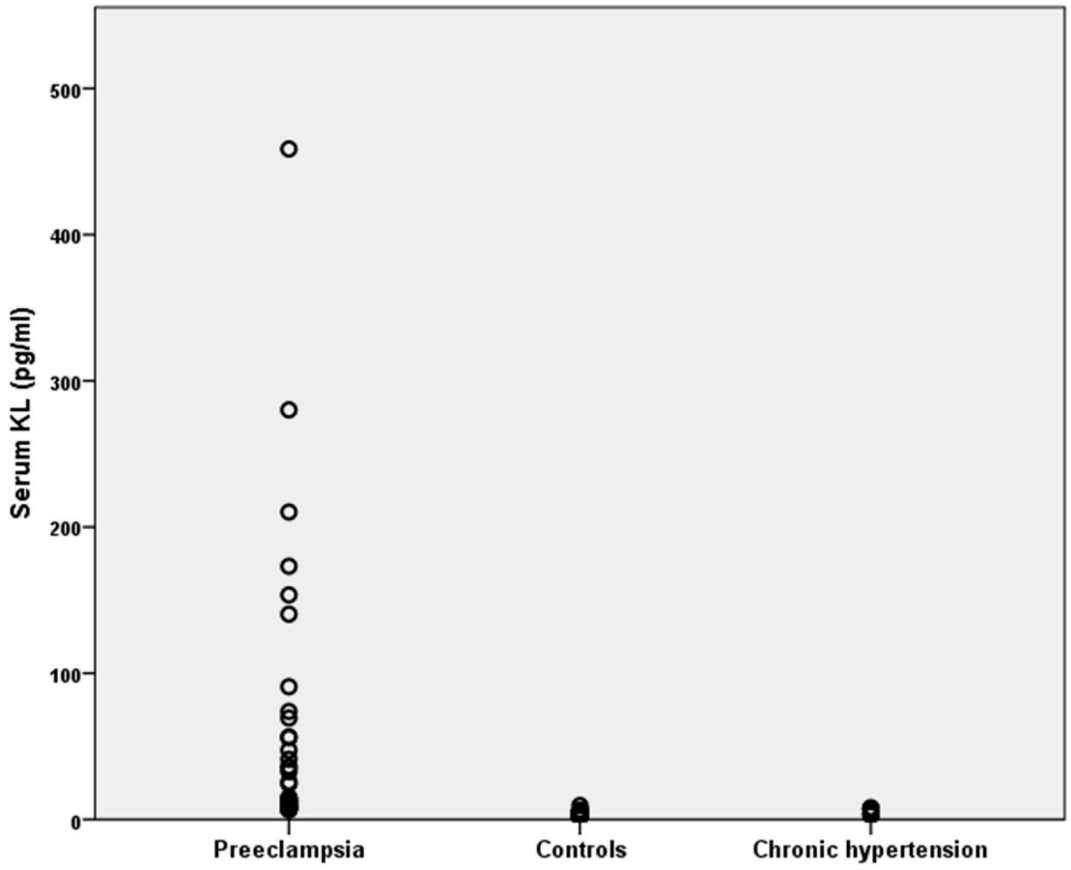

Fig. 1. Serum Klotho levels in preeclamptic, chronic hypertensive patients and healthy controls.

abnormal distributions. Mann-Whitney *U* test was used to compare variables that were not distributed normally. Kruskal-Wallis test was used for abnormally distributed continuous variables. When the Kruskal-Wallis test indicated significant differences, the causes of those differences were determined using a Bonferroni-adjusted Mann-Whitney *U* test. Analysis of covariance (ANCOVA) was used to test the differences of KL and DCN levels between groups, where gestational week at delivery was used as a covariate. Nominal variables were analyzed with a Pearson's chi-square or Fisher-Freeman-Halton exact test when applicable. A Spearman's correlation test was used to assess the correlations between the variables.

Receiver operating characteristic (ROC) curve analysis was used to determine the cut-off points for parameters. The diagnostic performance of KL and DCN measurements were evaluated, including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

A p value < 0.05 was considered to be statistically significant.

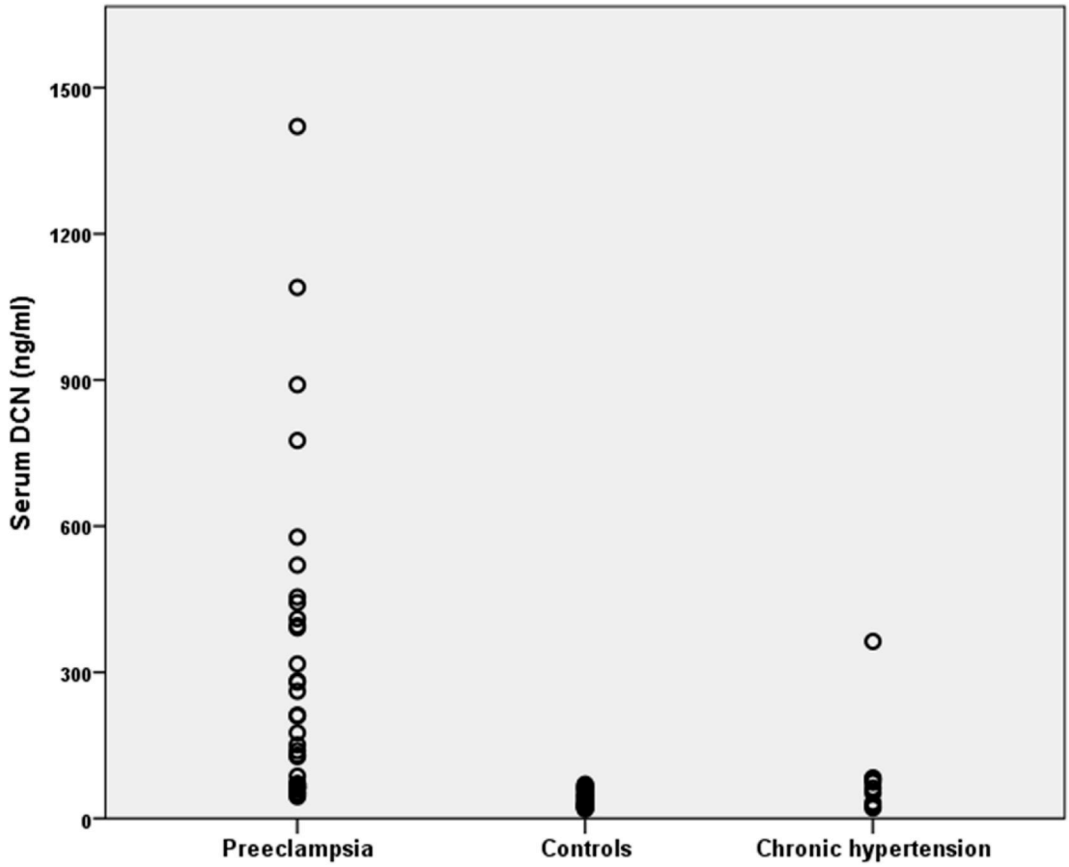

Fig. 2. Serum decorin levels in preeclamptic, chronic hypertensive patients and healthy controls.

Table 3
Serum Klotho and decorin levels (Mean ± SD).

	Human Klotho (pg/ml)	Decorin (ng/ml)
Preeclampsia	112.42 ± 172.78	292.46 ± 319.95
Controls	6.84 ± 3.45	40.93 ± 16.48
Chronic HT	9.86 ± 3.75	87.55 ± 99.76
	P	p
Preeclampsia *vs* Controls	0.006	0.001
Preeclampsia *vs* Chronic HT	0.043	0.041
Controls *v s* Chronic HT	0.999	0.999

HT: Hypertension.
ANCOVA with Bonferroni corrected post-hoc test.

4. Results

A total of 74 patients were included in the study. The demographic and clinical characteristics of the study population (severe preeclampsia [n:36], uncomplicated pregnancies [n:28], chronic hypertensive pregnancies [n:10] are displayed in Table 1.

A Bonferroni-adjusted Mann-Whitney *U* test indicated significant differences for body mass index (BMI) and weight between the groups. The BMI ($p = 0.008$; $p = 0.027$)and weight ($p = 0.005$; $p = 0.024$) were significantly higher in women with chronic hypertension than in women in the other groups.

The obstetric characteristics of the groups are shown in Table 2. Bonferroni-adjusted Mann-Whitney *U* test showed that gravidity ($p = 0.018$) and gestational age at delivery ($p = 0.005$) were both lower in preeclamptic patients than controls. As expected, IUGR was higher in preeclamptic patients ($p = 0.002$) compared to women with chronic hypertension and controls. The incidence of previous miscarriages was significantly higher in chronic hypertensive patients than in the other groups ($p = 0.03$).

Serum KL and DCN levels are shown in Figs. 1 and 2 in the three groups. Serum KL ($p = 0.006$; $p = 0.043$) and DCN ($p = 0.001$; $p = 0.041$) levels were significantly higher in women with PE than in controls and in women with chronic hypertension (Table 3).

By a ROC curve analysis, the best cut-off level for human KL to identify the presence of PE was calculated as 12.48 pg/ml with a sensitivity of 100% and specificity of 96%. The respective NPV and PPV were 100% and 97.3%. The area under the curve (AUC) was 99.1% with a 1% standard deviation (SD) and with an odds ratio of 37.00 (95%CI:5.35–255.75; Fig. 3).

Also, the best cut-off level for DCN was found as 62.33 ng/ml to assess the presence of PE witha sensitivity of 86.1% and a specificity of 88%. NPV was 81.5% whereas PPV was observed as 91.2%.The AUC was 94.1% with a 2.7% SD and with an odds ratio of 45,467 (%95 CI:9.825–210.413) (Fig. 4).

Placental KL levels were significantly higher in the patients with PE than in the healthy controls or in women with chronic hypertension ($p = 0.049$; $p = 0.029$; Table 4). Placental DCN was similar between preeclamptic women and healthy controls ($p > 0.05$; Table 4).

There was no significant difference in KL or DCN gene expression between the groups (Table 5). A weak correlation was observed between gestational weeks and serum KL levels ($r = -0.273$; $p = 0.021$). Serum KL levels decreased with gestational age. In the group analysis, this correlation was found as a significant variable only in the chronic hypertensive patients ($r = -0.645$; $p = 0.044$; $p < 0.05$). In the preeclamptic patients and healthy controls, gestational age was not a significant variable affecting KL levels ($p > 0.05$). Also, there was no correlation between BMI and serum levels of KL or DCN ($p > 0.05$).

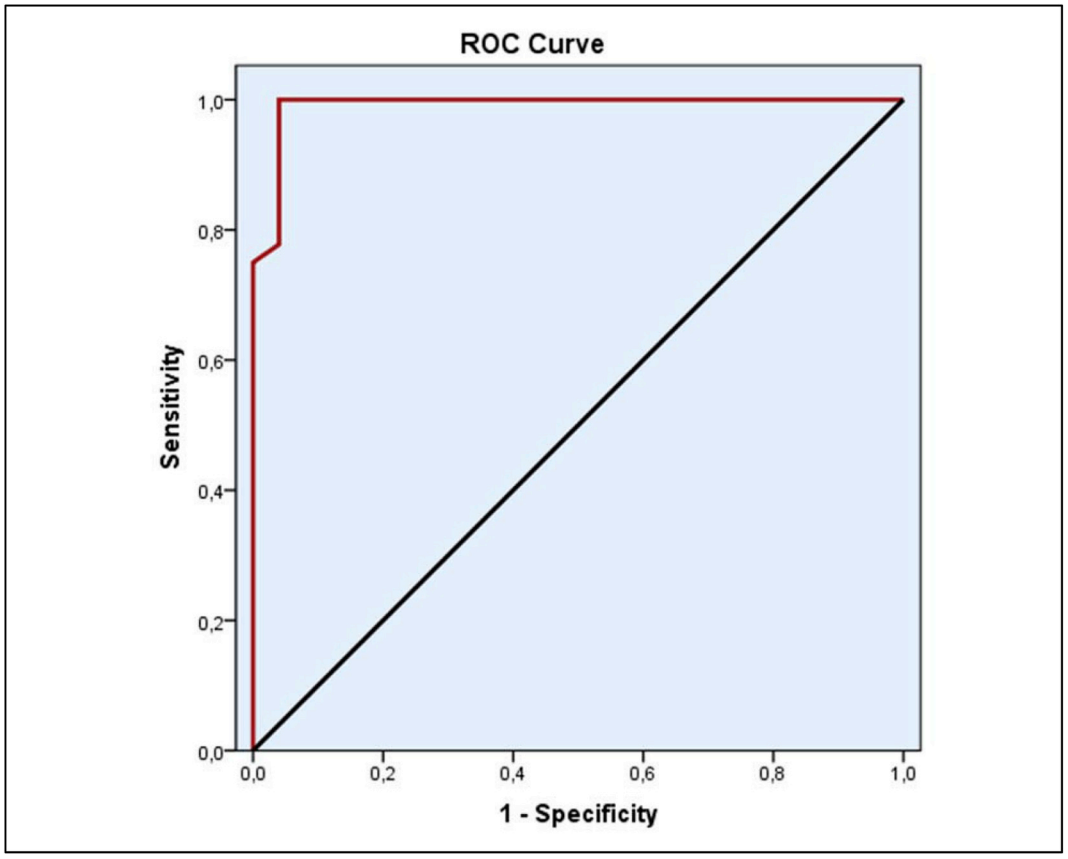

Fig. 3. ROC curve for serum Klotho concentrations in predicting severe preeclampsia; optimal cut-offvalue: 12.48 pg/ml (sensitivity 100%; specificity 93.3%).

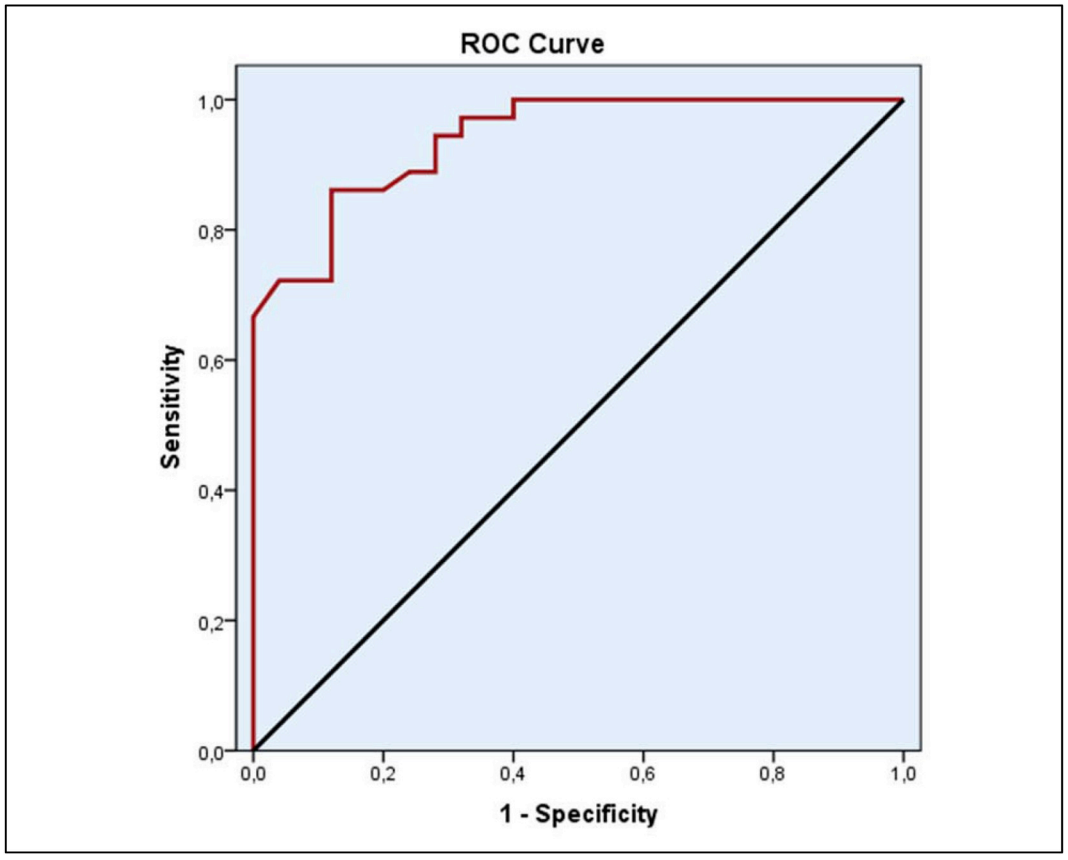

Fig. 4. ROC curve for decorin concentrations in predicting severe preeclampsia; optimal cut-off value: 62.33 ng/ml (sensitivity 86.11%; specificity 88%).

Table 4
Placental Klotho vedecorin levels in the groups (Mean ± SD).

	Klotho (pg/ml)	Decorin (ng/ml)
Preeclampsia	12.46 ± 12.48	28.97 ± 43.07
Controls	6.30 ± 4.90	16.91 ± 16.11
Chronic HT	3.01 ± 3.08	6.03 ± 5.03
	P	p
Preeclampsia *vs* Controls	0.049	0.455
Preeclampsia *vs* Chronic HT	0.029	0.181
Controls *vs* Chronic HT	0.999	0.999

HT: Hypertension.
ANCOVA with bonferroni corrected post-hoc test.

Table 5
Maternal blood Klotho and decorin gene expression.

	Klotho	Decorin
Preeclampsia	1.48 ± 1.46	17.65 ± 46.50
Controls	364.86 ± 1500.85	5009.50 ± 20640.65
Chronic HT	0.90 ± 0.67	38.54 ± 119.14
	P	p
Preeclampsia vs Controls	0.273	0.274
Preeclampsia vs Chronic HT	0.999	0.999
Controls vs Chronic HT	0.737	0.746

HT: Hypertension.
ANCOVA with bonferroni corrected post-hoc tests.

5. Discussion

In our study, we measured the KL precursor, human KL, in preeclamptic patients, pregnant women with chronic hypertension and women with uncomplicated pregnancies and found significantly increased serum and placental human KL in the preeclamptic group, but KL gene expression was not different in the preeclamptic patients than in the healthy controls. The best cut-off for KL for identifying the presence of PE was 12.48 pg/ml, with respective sensitivity, specificity, NPV and PPV were 100%, 96%, 100% and 97.3%. Placental KL was also found to be higher in preeclamptic patients than pregnant women with chronic hypertension or healthy pregnant controls. KL gene expression in maternal blood was similar in the groups. Thus, we can think that the source of KL in preeclamptic patients most likely is trophoblasts. Gestational age and BMI did not have any effect on maternal KL levels.

The human KL gene and its products are a new area of focus for PE and related diseases. It has many significant effects on endothelial nitric oxide production, angiogenesis, antioxidant enzyme production and protecting against endothelial dysfunction, all of which may be associated with PE [14–16]. The gene encodes three different forms with known subsequent functions. These are α-KL, β-KL and the KL-related protein (Klrp). Secreted α-KL has been found to be generated by alternative mRNA splicing [24,25]. Klrp is a transmembrane protein that binds to fibroblast growth factor (FGF) receptors [24]. The major human gene product is the secreted form, but the transmembrane protein is also shed into the blood [27]. ADAM10 and ADAM17 act as sheddase for the transmembrane protein. ADAM17 is also a major sheddase for placental TNF-α and increases in PE [28]. The transmembrane protein may also act in the pathogenesis of PE. KL proteins are also essential components of FGF receptor complexes as they are required for the high-affinity binding of FGF19, FGF21 andFGF23 [29]. The receptor for soluble α-KL is unknown, while the transmembrane form interacts with FGF isoforms [30]. Maternal serum FGF21 levels have been previously reported as increased in PE [31].

DNA methylation tests showed accelerated placental aging in early onset preeclampsia [32]. However the pathophysiologic mechanism of the placental aging is unclear. Since KL seems associated with anti-aging activities in mice [10], and PE is also associated with premature placental aging, we sought to investigate its serum and placental sample levels in PE. Previous studies on PE and KL have all been on soluble α-KL and have shown conflicting results. Consistent with our results, a recent study found higher systemic and placental α-KL levels in preeclamptic women [18]. On the contrary, some other studies observed lower soluble α-KL and α-KL gene expression in maternal serum and placenta in preeclamptic women compared to those with uncomplicated pregnancies [17,26]. KL seemed to decrease with gestational age in all patients; however, it was a significant variable only in chronic hypertensive pregnant women, in our study. The expression of KL in placenta, reproductive organs and other organs were not studied previously in preeclamptic patients in the literature. Since placental and serum Klotho levels were higher in women with preeclampsia, it can be hypothesized that placental expression of KL may increase in preeclampsia and placental KL may also indirectly regulate some target organs.

Higher levels of KL in the earlier weeks of pregnancy may lead to believe that human KL can be utilized as a potential biomarker to predict PE in the early weeks of the pregnancy, but larger prospective randomized studies are needed to clarify this hypothesis. According to our findings, KL also seems to be a prognostic candidate as a potential biomarker because of its high sensitivity and NPV in the presence of PE.

In our study, we found maternal plasma human KL levels were 112.42 ± 172.78 pg/ml in PE patients, 9.86 ± 3.75 pg/ml in chronic hypertensive pregnant women and 6.84 ± 3.45 pg/ml in healthy pregnant controls. A previous study that measured soluble α-KL levels found that mean ± SD soluble α-KL was 2019 ± 1320 pg/mL in patients with PE and 1277 ± 909 pg/mL in the controls [18]. However, in our study, human KL levels were ten-fold higher in PE patients compared to controls, and thus we can assert that human KL seems to be a more sensitive marker than soluble α-KL for women with PE.

We also examined the DCN levels in the serum and in the placenta, together with the gene expression in women namely with pregnancy induced maternal blood, in different pregnant hypertension, chronic hypertension and healthy pregnant patients. A leucine-rich proteoglycan, DCN has been suggested as a potential biomarker for PE by its role in the possible pathophysiologic mechanisms in the development of PE. DCN limits the invasion and endovascular differentiation of trophoblast cells by binding to tyrosine kinase receptors and VEGF receptor-2 [19,33]. Other than well-known markers for preeclampsia, we have chosen DCN, because DCN dysregulation and DCN gene polymorphisms were also blamed in renal diseases and appear to have biological relevance in human vascular pathophysiology [34,35].

In our study, we found that DCN was higher in patients with PE than in pregnant women with chronic hypertension and healthy pregnant controls. For DCN, sensitivity, specificity, NPV and PPV for the detection of PE were 86.1%, 88%, 81.5% and 91.2, respectively. Our results for serum DCN levels in the preeclamptic patients were less sensitive than those for KL. In the placental or DCN gene expression analysis, DCN was similar in patients with PE and in the controls.

We can conclude that human KL seems as a potential marker for PE, with high sensitivity and specificity. It also appears to be more sensitive and specific than human DCN, which has been studied in association for PE for many years. Since PE is a placental originated disease and human KL may work in different pathways associated with PE, we have demonstrated that placental human KL was increased in PE patients. Therefore, human KL may also help us to understand the pathophysiologic mechanisms of PE better. Molecular studies are needed to clarify the role of human KL in the presence of PE. Prospective randomized clinical studies those are to be done before the onset of PE may also enlighten the feasibility of human KL as a predictor of PE in the earlier gestational weeks.

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Conflicts of interest

The authors have no conflicts of interest in any of the products mentioned in this article.

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