Correlation of Cytotoxic T-Lymphocyte-Associated Protein 4 and CD80 with Some Trace Elements in Preeclampsia.

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Abstract
Preeclampsia (PE) is a complicated illness defined by hypertension and proteinuria after the 20th week of pregnancy, and it is the leading cause of infant morbidity and death. It is associated with many biochemical and immunological disorders. However, it is not clear what type of inflammation is involved in the onset of PE. In the present study, the serum levels of CD80 and its receptor cytotoxic T lymphocyte-associated antigen-4 (CTLA4), vitamin D (VD), and Granulocyte-macrophage colony-stimulating factor (GM-CSF) have been measured in 90 PE women and compared with 30 healthy pregnant women. The levels of these biomarkers were correlated with some cations and trace elements. The results showed an increase in CD80, CTLA4, GMCSF, and copper in PE women compared with controls. While a lower VD, and zinc were found in patients' group. The receiver-operating characteristic (ROC) analyses validated the low VD and high CTLA4 for the diagnosis of PE in suspicious persons with good sensitivity and specificity. The overall conclusions of the present study are the presence of a state of inflammation, and deficiency of major micronutrients in PE women compared with healthy controls that need to be corrected.

Keywords: Preeclampsia, CD80, cytotoxic T lymphocyte-associated antigen-4, vitamin D, Granulocyte-macrophage colony-stimulating factor, copper, and zinc.

1. Introduction

Preeclampsia (PE) is a complication of pregnancy involving more than one bodily system; it is common and exposes the mother or the child to increased risk for morbidity and mortality. Clinically presented with two main characteristics: new-onset HTN and proteinuria that occurs after week 20 of gestation or after delivery. Also, it could involve new-onset PE-related signs but without proteinuria [1]. PE may have a wide range of symptoms and attempts to subclassify the condition based on start time have had mixed results. The main reason behind PE is not yet well defined; however, immune system involvement has been pointed to recently by several studies and data, and defective conceptus tolerance is a fundamental part of the pathogenesis, especially in cases of early-onset and severe PE [2]. Eventually, it ends in enormous system inflammation with activated circulating T cells [3, 4]. Common signs of PE include headaches, vision problems, and abdominal discomfort; however, it may be recognized before symptoms start by monitoring blood pressure and weight increase. Several researchers have found a drop in serum albumin levels during a typical pregnancy. In individuals suffering from severe toxemia during pregnancy, all of the distinct fractions of plasma protein decrease in concentration [5]. Cytotoxic T-lymphocyte antigen-4 (CTLA4) or CD152 is a type of protein receptor that works as an immunity checkpoint and downregulates immunological responses [6]. A panel of immune-modulatory receptors expressed on the Treg cell population includes CTLA4 that is found on activated T and Treg cells [7]. It serves as a negative regulator of the activation of T-cell [8]. It may act in vivo through the capture and removal of CD80 and CD86 from antigen-presenting cells membranes, as a result it makes them unavailable for CD28 triggering [9]. When attached to CD80 or CD86, which are both expressed on antigen-presenting cells surface, it functions as an "off" switch [9, 10]. B7-1, a B cell activation molecule is identified for as a ligand for CTLA4 [11].

Vitamin D is important for reproductive success, like attaining a pregnancy by means of assisted reproductive technology [12]. When levels of VD has been compared between PE patients and control group both at mean gestation; week 14, VD level was significantly lower in PE patients group. Leading to conclusion that lower VD levels expose women to higher risk of PE [13, 14].

The study was intended to determine the ability of specific biomarkers (CD80, CTLA4, GMCSF, and VD) to aid the diagnosis and prognosis of PE in addition to routinely measured parameters. The study aims to detect the correlation of CTLA4, CD80, and granulocytes macrophage stimulating factor, and VD with some trace elements in PE.

2. Subjects and Methods

Subjects

In the present research, there were 90 PE patients with an average age of 32.67±5.88 years old and thirty healthy pregnant controls with an equivalent age and gestational age collected from a maternity teaching hospital and some private clinics and hospitals from October 2021 to May 2022. In order to make a

doi.org/10.31838/hiv22.02.358 Received: 11.04.22, Revised: 11.05.22, Accepted: 18.08.22

conclusive diagnosis of PE, the standards established by the American College of Obstetricians and Gynecologists were followed [15, 16]. After 20 weeks of pregnancy, a woman is said to have PE if she has protein in her urine and her BP is higher than 140/90 millimeters of mercury on the systolic and diastolic scales, respectively. All of the patients in this research fulfilled these requirements, and their dipstick tests indicated the presence of proteinuria in all of them. They also received therapy with methyldopa (Aldomet®) and fasted for the previous night. The ethical approval committee of the University of Hawler, Erbil, Iraq (Document number 103/2022) gave their stamp of approval to the research project, ensuring that it is in line with the standards outlined in the "International Guideline for Human Research" that the Declaration of Helsinki mandates.

As a control group, thirty age-matched, normotensive pregnant women who were more than 20 weeks along in their pregnancies and showed no obvious abnormalities were chosen. No one of the subjects experienced active diseases like uterine contractions or membrane ruptures in the recent past. In order to rule out the possibility of any overt systemic inflammation, the serum C-reactive protein (CRP) levels in all of the samples came back negative, coming in at less than 6 mg/l.

Methods

After an overnight fast, five milliliters of venous blood samples were drawn from each patient using disposable needles and plastic syringes. The samples were moved to a fresh tube with no markings. Hemolysis samples were thrown away. The blood was allowed to coagulate for 15 minutes at room temperature, centrifuged at 3000 rpm for 5 minutes, and then the serum was separated and transferred to fresh disposable Eppendorf tubes.

The CD80, GM-CSF, TCLA-4, and VD were estimated using an enzyme-linked immunosorbent assay (ELISA). Estimating serum Cu and Zn was done using the colorimetric method, and CRP was measured using the serology qualitative technique.

Statistical Analysis

The Kolmogorov-Smirnov test was used as a numerical means of assessing normality. Analysis of Variance (ANOVA) test was employed to assess differences in scale variables between diagnostic groups. Analysis of contingency tables (Chi-square, $\chi 2$ test) was used to check the comparison between the categorical variables. The results were expressed as mean±standards deviation for normally distributed values and 25-75% percentile (median) to express the results of nonparametric variables. Pearson's correlation coefficients were used to examine associations between scale variables and Spearman's correlation coefficient (rho, ρ) to find the correlation between nonparametric parameters and other variables. Multivariate general linear model (GLM) analysis was used to assess the effects of diagnosis (independent variable) on biomarkers and scales (dependent variables) while adjusting for extraneous variables (age, BMI, BP, parity, and sex). Tests between subjects were performed to show the effect of diagnosis on the measured biomarker after adjusting for all confounders. The effect was estimated by the partial eta squared ($\eta 2$) function. ROC curves were measured to examine the diagnostic ability of the measured biomarkers to diagnose the disease and abortion. All statistical analyses were performed using SPSS windows version 25, 2017.

3. Results

The results of demographic and clinical data on healthy controls (HC) and PE patients are presented in Table 1.

Table 1: Demographic and clinical data of healthy controls (HC) and PE subjects

Parameter	Patient	Control	F/$\chi 2$	p
Age yrs.	32.67±5.875	31.37±6.026	1.088	0.299
Weight kg	85.46±15.163	86.37±10.656	0.093	0.761
Height cm	164.20±4.918	166.87±7.385	2.056	0.056
BMI kg/m2	31.7069±5.338	30.97462.941	0.511	0.476
Rural/Urban	40/50	14/16	0.045	0.836
Education yrs.	6.20±3.073	6.10±3.346	0.023	0.880
Smoking No/Yes	87/3	29/1	0.001	1.000
Gestational age Wks.	30.10±4.389	29.60±4.882	0.276	0.600
Systolic B.P. mm/Hg	0.557±15.014	120.63±2.109	161.990	<0.001
Diastolic B.P. mm/Hg	91.11±9.978	80.37±2.566	33.861	<0.001
Previous abortion Yes/No	38/52	25/5	15.249	<0.001
Nullipara (no live births)/multipara	15/75	4/26	8.944	0.005
Cesarean delivery Y1,N0	37/53	21/9	7.519	0.011
Gravidity (#Preg.)	3(2-4)	3(2-5)	1.976	0.133
Parity (#Deliv)	2(1-3)	3(2-4)	MWUT	0.033
Age of Onset yrs.	29.29±5.228	-	-	-
Duration of PE yrs.	3.38±3.056	-	-	-
Symptoms Duration Wks.	4.284±8.7584	-	-	-
CTLA4 pg/ml	639.002 (544.469-748.813)	371.900 (310.140-422.365)		<0.001
CD80 pg/ml	467.454 (384.345-568.493)	340.935 (288.792-36.797)		<0.001
VD ng/ml	7.960 (6.822-9.237)	11.060 (10.215-12.880)		<0.001
GMCSF pg/ml	130.591 (100.919-167.284)	97.505 (71.870-116.057		<0.001

Results are expressed as mean ± standard deviation for normally distributed data. Binomial data were expressed as ratios and analyzed by the Chi-squared test. BMI: body mass index, B.P.: blood pressure.

The results indicated that age, BMI, education level, smoking status, number of pregnancies, gestational

age, and ratio of nulliparas to multiparas have insignificant variation between PE patients and the control group. When compared with healthy pregnant women, the PE women exhibited a substantial rise in the number of prior abortions and Caesarean births, and the parity of patients with PE (number of children born) is quite less than in control groups.

The results showed a significant increase ($p<0.001$) in serum CTLA4, CD80, GMCSF, and CTLA4 in PE patients compared with the healthy controls. Serum VD levels showed a significant decrease ($p<0.001$) in PE patients as compared with the healthy controls group.

The results of the serum level of Cu, and zinc, and their ratio in HC and PE patients are presented in Figure 1. The results showed a significant increase in serum Cu ($p=0.028$) and Cu/Zn ratio ($p<0.001$) in PE patients compared with the control group. In contrast, serum Zn in PE patients is significantly decreased ($p<0.001$) compared with healthy pregnant women.

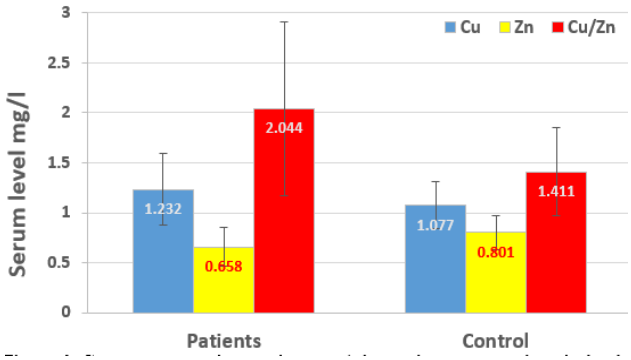

Figure 1: Serum copper, zinc, and copper/ zinc ratio concentrations in healthy controls and PE patients.

The correlations between the measured biomarkers VD, CD80, CTLA4, and GMCSF in the demographic and clinical parameters are presented in Table 2 and showed significant inverse correlations of VD with the age of onset, systolic and diastolic BP duration of symptoms and disease, nullipara, and history of abortion and cesarean delivery. There are significant inverse correlations between VD with copper and Cu/Zn. CTLA4 is significantly and inversely correlated with serum zinc and Cu/Zn. At the same time, GMCSF and CD80 have no significant correlation with the measured trace elements. The results showed a significant negative correlation between VD with all other biomarkers (CD80, CTLA4, and GMCSF). CTLA4 has a significant correlation with CD80.

The multiple regression analysis (Table 3) was used to determine the cofounders' effect on all measured parameters. The results showed that the diagnosis (presence of PE in a subject) is the only cofounder that significantly affects ($p<0.001$) the level of the measured biomarkers with a high size of the effect (Partial $\eta 2=0.654$). Therefore, we used the diagnosis only as an explanatory factor to explain its effect on the measured biomarkers. Tests for between-subjects effects show that the top three parameters that have been significantly affected by diagnosis are CTLA4 (Partial $\eta 2=0.339$), VD (Partial $\eta 2=0.212$), and CD80 (Partial $\eta 2=0.092$).

Table 2: Correlation of the biomarkers with demographic parameters

Parameters	Vitamin D	CTLA4	GMCSF	CD80
Age	-0.147	0.065	0.112	-0.013
Age of Onset	-0.464**	0.527**	0.298**	0.277**
Duration of PE	-0.599**	0.432**	0.271**	0.308**
Weight	-0.033	-0.201	-0.153	-0.073
Height	0.102	-0.152	-0.153	-0.056
BMI	-0.116	-0.107	-0.077	0.019
Systolic B. P	-0.466**	0.655**	0.232*	0.097
Diastolic B. P	-0.393**	0.431**	0.325**	0.242**
Gestational age	0.023	-0.104	-0.047	0.129
Duration Symptoms	-0.458**	0.433**	0.331**	0.463**
Education	0.001	-0.078	0.110	0.057
Residency	-0.021	0.085	0.139	0.099
Gravidity (#Preg.)	0.099	-0.184	-0.206*	0.041
Previous abortion	-0.225*	0.249**	-0.005	0.111
Nullipara/multipara	-0.219*	0.084	0.079	0.115
Smoking	-0.103	-0.063	0.130	0.121
Cesarean delivery	-0.246**	0.044	0.024	0.137
Parity (#Deliv)	0.124	0.159	0.058	0.114
Zinc ppm	0.178	-0.225*	-0.068	-0.116
Cu ppm	-0.194*	0.126	0.115	0.160
Cu/Zn	-0.243**	0.224*	0.097	0.152
Vitamin D	1.000	-0.332**	-0.269**	-0.329**
CTLA4	-0.332**	1.000	0.262**	0.262**
GMCSF	-0.269**	0.085	1.000	0.138
CD80	-0.329**	0.262**	0.138	1

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Table 3: Results of the multivariate generalized linear model (GLM) analysis and the between-subjects effects of the effect of the diagnosis on the biomarkers.

Tests	Dependent Variables	Explanatory variables	F	P	Partial η2
Multivariate	Biomarkers	Diagnosis	13.981	<0.001	0.654
		Gravidity	1.741	0.064	0.191
		Gestational age	1.365	0.191	0.156
		Smoking	1.080	0.385	0.128
		Residency	1.034	0.426	0.123
		BMI	1.024	0.435	0.122
		Abortion	0.916	0.540	0.110
		Cesarean delivery	0.911	0.545	0.110
		Duration of symptoms	0.809	0.649	0.099
		Parity	0.811	0.633	0.091
		Age of Onset	0.618	0.833	0.080
		Age	0.528	0.902	0.067
		Duration of PE	0.505	0.917	0.064
		Nullipara	0.318	0.988	0.043
Tests for between-subject	CTLA4	Diagnosis	55.357	<0.001	0.339
	Vitamin D	Diagnosis	29.044	<0.001	0.212
	CD80	Diagnosis	10.995	0.001	0.092
	Cu/Zn	Diagnosis	6.733	0.011	0.059
	Zinc	Diagnosis	5.445	0.021	0.048
	GMCSF	Diagnosis	4.935	0.028	0.044
	Cu	Diagnosis	2.098	0.15	0.019

To determine the diagnostic sensitivity and specificity of the measured biomarkers for the diagnosis of PE, an analysis of (ROC) was performed. The ROC curves of the analysis are plotted in Figure 2. At the same time, the coordinates of the ROC results and the cut-off of the concentration that produce the best sensitivities and specificities are presented in Table 4.

Table 4: Receiver operating characteristic-area under curve (AUC) analysis of the measured biomarkers for the diagnosis of PE. CI: Confidence interval.

Variable	Cut-off Level	Sensitivity %	Specificity %	Youden's Statistics	AUC	95% CI of AUC	p-value
CTLA4 pg/ml	462.61	91.1	90.1	0.78	0.939	0.898-0.980	<0.001
Vitamin D ng/ml	9.88	86.7	83.3	-0.76	0.921	0.887-0.978	<0.001
CD80 pg/ml	399.22	68.9	66.7	0.50	0.766	0.654-0.878	<0.001
GMCSF pg/ml	109.01	68.9	67.6	0.44	0.761	0.666-0.856	<0.001

The results showed an increase in CTLA4 higher than the cut-off value (462.61 ng/ml) indicates that the subjects may have a PE with a sensitivity and specificity of 91.1% and specificity of 90.1%, respectively. CTLA4 curve covers a high AUC with a high Youdin J statistic (0.78), respectively. The decrease in VD lower than the cut-off value (9.88ng/ml) leads to the diagnosis of a subject with PE with a sensitivity of 86.7% and specificity of 83.3%. VD curve covers a high AUC. The diagnostic characteristics of CD80 and GMCSF were relatively low.

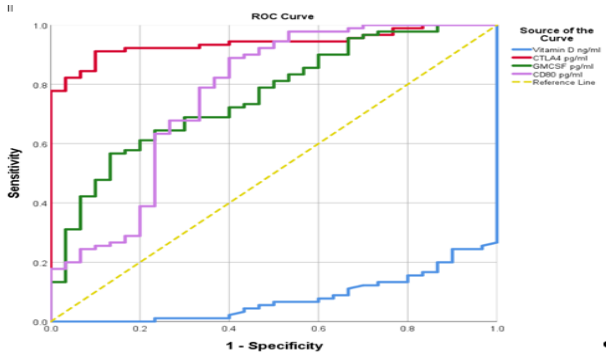

Figure 2: Receiver operating characteristic curves of VD, CTLA4, CD80, and GMCSF for diagnosing PE against healthy controls.

4. Discussion

The main results in Table 1 regarding the comparison between demographic and clinical data are the substantial rise in the number of prior abortions, cesarean births, and blood pressure levels in PE patients compared with controls. At the same time, there is a lower parity (the number of children born) in patients with PE compared with the healthy group. Serum CTLA4 in PE patients was greater than in healthy controls, indicating the immune system's involvement in the pathophysiology or etiology of PE disease. Several signaling pathways seemed to have a crucial effect on the expression of CTLA. When animals were treated with CTLA4 blocking antibody, the fetal loss was shown to be greater with cytokine profile changes by decidual CD4+ T (dCD4+ T) cells [17]. Decidual Treg showed higher levels and a higher ratio to CTLA4 (both intracellular and surface) [18]. The higher serum CD80 in PE patients than in control indicates a role for the cells that synthesize CD80 or respond to the CD80-dependent signals. Previous research showed that after CTLA4 binds to its ligands CD80/CD86, it promotes their direct endocytosis through its tail part, resulting in CD80/CD86 down-regulation on APCs [19]. Lower VD in PE women compared with healthy pregnant women indicated a decrease in the main functions of VD in PE women, including its role in calcium and phosphate metabolism and as an immunity regulator [20]. Many studies showed the correlation between PE and low VD levels, making it preferable for women with a higher tendency to develop PE to take VD routinely [21]. According to growing data, VD shortage may be linked to recurrent pregnancy loss and poor obstetrical and neonatal outcomes [22].

The higher GMCSF serum levels in PE patients compared to normal pregnant women under previous papers [23]. Interaction of GMCSF with its receptor on Tregs may improve immune tolerance [24]. Also, GMCSF regulates the effector differentiation of natural killer T-cells, which express the receptors of GMCSF [25].

PE patients have notable higher serum levels of copper and Cu/Zn ratio, while there is a significant increase in serum zinc compared with controls. Different data proposed that essential trace elements have a fundamental effect on the development of PE, for example, zinc, magnesium, iron, calcium, and coppe [1, 26].

The results in Table 2 showed significant inverse correlations of VD with the age of onset, systolic and diastolic BP, duration of symptoms and disease, nullipara, and history of abortion and cesarean delivery. CTLA4, in Table 2, showed significant correlations with the age of onset, systolic and diastolic BP, disease duration, parity and nullipara/multipara ratio. These results indicated the role of CTLA4 as an immune stimulation biomarker in the clinical symptoms of PE patients.

The inverse correlations between VD with copper and Cu/Zn are presented in Table 2. Also, CTLA4 is significantly and inversely correlated with serum zinc and its ratio Cu/Zn. At the same time, GMCSF and CD80 have no significant correlation with the measured trace elements. The inverse correlation may be because zinc may enhance regulatory T cell numbers, while TCLA-4 has a suppressive effect on T-reg after binding with its receptor [27]. The significant negative correlation between VD and CD80, CTLA4, and GMCSF (Table 2) indicated a significant negative effect of the VD deficiency on

immunity. CTLA4 has a significant correlation with CD80, and this result is expected as CD80 is a receptor of CTLA4, and both are important regulators of the immune system [28].

Multiple regression analysis assessed the cofounders' effect on all observable parameters. The most significant factor that influenced biomarkers (PE diagnosis) was utilized as an explanatory factor to determine the effect of each biomarker after correcting for other cofounders using between-subjects analysis to estimate the effect size of each biomarker by the diagnosis. This was done to investigate the link between biomarkers and PE (presence of PE in a subject).

The ROC curve of the diagnosis of PE in Figure 2 and the analysis data in Table 4 indicated that the increase in CTLA4 and the decrease in VD are the best indicators for diagnosis of PE because they have the highest sensitivities and specificities. Increased parathyroid hormone concentration in conjunction with VD deficiency in advanced pregnancy stages puts mothers at a two-fold higher possibility of developing PE. In examining interactions between VD and known risk factors for PE and pregnancy-induced hypertension, maternal placental growth factor (PlGF) levels were insignificant in early and late second trimesters of pregnant women with diminished VD levels [29].

5. Conclusions

From the results of the present study, it can be concluded that PE women suffered from hypovitaminosis D. There is a significant increase in CD80, CTLA4, copper, and GM-CSF levels in PE women compared with healthy pregnant women. The study's overall conclusion indicated a state of inflammation and deficiency of major micronutrients in PE women compared with healthy controls that need to be corrected.

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