

Serum Interleukin-4 and Interleukin-10 Levels as Biomarkers in the Early onset Pre-eclampsia

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Abstract

Objective: To explore the association of IL-4 and IL-10 levels as biomarkers with EOP.

Methodology: It was a case-control study was conducted in King Edward Medical University (KEMU), Lahore from May 2020 to May 2021. Fifty five diagnosed patients with early onset pre-eclampsia as cases, and 56 normotensive pregnant controls were taken from Lady Atchison Hospital, Lahore. While, 32 non-pregnant controls from general population were enrolled in the study. Blood was collected from the subjects, and serum levels of IL-4 and IL-10 were determined by sandwich ELISA assay in the advanced research lab of biomedical sciences, KEMU, Lahore. Data was analyzed using SPSS version 26.0. The chi-square test was used to examine the association between categorical variables. To ascertain the discriminative abilities of interleukin-4 and interleukin-10, a receiver operating characteristic (ROC) curve analysis was performed. A p value of <0.05 was considered statistically significant.

Results: Women with EOP had a positive previous history of pre-eclampsia ($p < .001$). Significantly higher serum IL-4 levels were present in non-pregnant controls, followed by EOP and pregnant controls ($p < .001$). IL-10 levels were significantly lower in EOP and in pregnant controls as compared to non-pregnant controls ($p < .001$). IL-4 at a cut-off value of >11.6 (AUC=0.902, $p < .001$) had 87.27% sensitivity and 92.86% specificity to differentiate the patients with EOP from normotensive pregnant females.

Conclusion: Levels of serum IL-4 but not of IL-10 can be used as an independent biomarker to differentiate between early onset pre-eclampsia and normotensive pregnancy.

Keywords: Pre-eclampsia, early-onset pre-eclampsia, interleukin-4, interleukin-10, IL-4, IL-10, normotensive pregnancy, biomarker.

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Introduction

Preeclampsia (PE) typically occurs after the 20th week of pregnancy in a formerly normotensive female and is accompanied by either proteinuria, dysfunction of one or more maternal organs, or the presence of utero-placental dysfunction.¹ Pakistan has the world's third-highest rate of maternal and fetal mortality, with PE and its complications accounting for 34% of maternal deaths among those presented in tertiary care facilities.²

Apart from clinical risk factors, certain protein biomarkers such as pregnancy-associated plasma protein-A³ and soluble fms-like tyrosine kinase

receptor-1 have contributed towards understanding the pathogenesis of PE. Although combining clinical risk variables with these biochemical indicators increases PE's prognostic ability, more clinical studies are needed to validate these findings and to date PE is diagnosed after the onset of symptoms.⁴

The etiology of early onset preeclampsia (EOP) occurring on or before the 34th week of pregnancy, could be divided into two stages. At first stage, defective placentation, placental ischemia, and oxidative stress occur early in the first trimester. In this stage, there is a release of several anti-angiogenic

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factors, cytokines, and oxidants into the maternal circulation, leading towards stage II of systemic inflammation.⁵ There is a shift from the T-helper (Th)2 immune response towards the Th1 immune response in the later stage. This immune system dysregulation is responsible for the onset of clinical symptoms of EOP.⁶

IL-4 acts as a pleiotropic cytokine and is essential for the maturation of naive helper T cells into Th2 cells to generate a Th2 immune response.⁷ Similarly, IL-10 exerts a potent anti-inflammatory response by suppressing macrophage activity, and immune system dysregulation due to IL-10 deficiency, is implicated in the pathophysiology of EOP.⁸ The role of IL-10 and IL-4 in preeclampsia has shifted from a biomarker to therapeutic potential, as treatment with these cytokines restored spiral artery damage and PE like symptoms in pregnant mice.⁹ Furthermore, during endothelial dysfunction, serum cytokine levels are altered before the onset of clinical symptoms, and at this stage identifying their association with EOP may prove useful in early diagnosis. There hasn't yet been any research done in a developing country like Pakistan where levels of these potential biomarkers have been assessed, let alone in pregnant or preeclamptic females. The role of IL-4 and IL-10 was examined in our population for the first time in both normotensive pregnancy and EOP.

Methodology

This was an observational case-control study that adhered to the checklist provided by STROBE guidelines for reporting case-control studies.¹⁰ Women of reproductive age (between 15 and 49 years) were included in three study groups. A total of 55 patients with a diagnosis of EOP as cases and 56 gestational age-matched normotensive pregnant females as pregnant controls were recruited from the Department of Obstetrics and Gynecology, Lady Aitchison Hospital, Lahore. According to the diagnostic criteria of the International Society for the Study of Hypertension in Pregnancy, EOP was defined as "systolic or diastolic BP >140 mm Hg or 90 mm Hg, respectively, with significant proteinuria (>300 mg/24 hr or ≥1+ on urine dipstick test) between the 20th and 34th weeks of gestation in a previously normotensive woman".¹

While, normotensive pregnant females were defined as having BP <140/90 with no proteinuria. Moreover, 32 non-pregnant females of reproductive age with BP <140/90 and no proteinuria were also taken from the general population as non-pregnant controls. Subjects were recruited from May 2020 to May 2021. Subjects

with a history of systemic inflammatory disease or autoimmune disorder, including chronic hypertension, diabetes mellitus, glomerulonephritis, hepatitis, systemic lupus erythematosis, rheumatoid arthritis, gestational diabetes or twin pregnancy, were excluded from the study. Furthermore, women receiving steroids or any other medications that alter the immune system's response were excluded as well. A non-probability, convenient sampling technique was used for the selection of patients.

In our study, 143 female subjects were included in three study groups. The sample size was calculated using 95% power of the test and 5% level of significance, with expected mean value of IL-10 in cases as 4.34 ± 1.53 pg/ml and in pregnant controls as 7.61 ± 3.2 pg/ml.¹¹

All guidelines for the use of human biological samples were followed in this research project. By letter number. 946/RC/KEMU dated 13/07/2019, the institutional review board of King Edward Medical University (KEMU), Lahore, approved the study. The subjects gave their written, informed consent at the time of recruitment. After obtaining demographic and clinical data of participants, each individual had a 2-3 ml blood sample taken using clot separator gel in a dry vacuum tube. To separate the serum, blood sample vials were centrifuged at 1000 rpm for 10 minutes and after centrifugation, serum was stored at -80°C in sterile microcentrifuge tubes until further use.

Levels of interleukin-4 and interleukin-10 were estimated in the advanced research lab of biomedical sciences, KEMU, using standard IL-4 and IL-10 ELISA kits. The kits were provided by Thermofisher Scientific Inc. (Waltham, MA, USA) having Catalogue numbers BMS225-2 and BMS215-2 for IL-4 and IL-10, respectively. The IL-4 ELISA kit had an assay range of 7.8-500 pg/mL and an analytical sensitivity of 1.3 pg/mL, with intra-assay and inter-assay coefficients of variation of 4.8% and 5.6%, respectively. The IL-10 ELISA kit had an assay range of 3.15-200 pg/mL and an analytical sensitivity of 1.0 pg/mL, with intra-assay and inter-assay coefficients of variation of 3.2% and 5.6%, respectively. The mean recovery of the cytokine in serum for IL-4 and IL-10 was 94% and 97%, respectively. No considerable cross-reactivity or interference with immune system circulating components was present in cytokine-positive serum. In the above-mentioned ELISA kits, the manufacturers protocol was followed. At a wavelength of 450 nm,

standard curves were plotted on graphs automatically by AccuSkan FC micro-plate reader (Fisher Scientific, Pittsburgh, Pennsylvania, USA) for each cytokine, and results were read from the curves for each sample.

Data was analyzed using SPSS version 26.0. Shapiro-Wilk and Kolmogorov-Smirnov tests were employed to verify that continuous data was normal. The analysis of variance (ANOVA) test was used to analyze normally distributed continuous variables. Continuous variables with a non-normal distribution were compared with the Kruskal-Wallis test. The chi-square test was used to examine the association between categorical variables. To ascertain the discriminative abilities of interleukin-4 and interleukin-10, a receiver operating characteristic (ROC) curve analysis was performed. A p value of <0.05 was considered statistically significant.

Results

The demographic and disease-related variables of the subjects are given in Table I. Levels of IL-4 and IL-10 were within the detection limit of ELISA kits in all non-pregnant females. But IL-4 was detected in 28.6% of pregnant controls and in 89.1% of preeclamptic patients (p <0.001). Whereas, only 12.7% of EOP patients and 16.1% of pregnant controls had detectable IL-10 levels (p <0.001) (Figure 1).

By following the statistical analysis of Ten Berge *et al.*, values less than lower limit of detection were substituted with the lowest value of the reference curves¹² (i.e., 7.79 for IL-4 and 3.14 for IL-10), and serum cytokines were compared using the Kruskal-Wallis test. IL-4 levels were significantly higher in non-pregnant controls and in patients with EOP as compared to pregnant controls, and the difference was statistically significant in all study groups (p<.001).

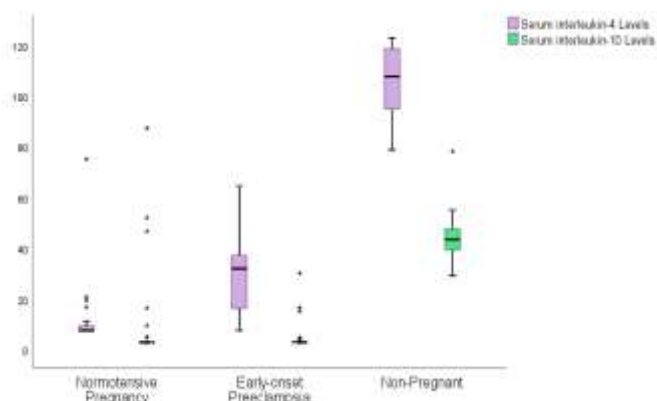


Figure 1. Box plot of serum IL-4 and IL-10 levels in study groups.

Similarly, IL-10 levels were significantly lower in the patients of EOP and in pregnant controls as compared to non-pregnant females (p<.001), but the difference in the levels was statistically not significant between EOP and pregnant controls (p=0.68) as shown in Table II.

The ability of IL-4 and IL-10 levels to discriminate hypertensive and normotensive pregnancies was assessed using ROC curve analysis. At a cut-off point of >11.6 (p<.001), the area under the curve (AUC) for IL-4 to discriminate early-onset preeclampsia from normotensive pregnancy was 0.902. For IL-10 the AUC was 0.511 with a p-value of 0.729 at a cut-off point of ≤3.14 (Figure 2).

The values of the validation criteria for differentiation between normotensive pregnancy and women with early-onset pre-eclampsia for the respective cut-off values of interleukin-4 and interleukin-10 are given in Table II. The values of the validation criteria for differentiation between normotensive pregnancy and women with early-onset pre-eclampsia for the respective cut-off values of interleukin-4 and interleukin-10 are given in Table III.

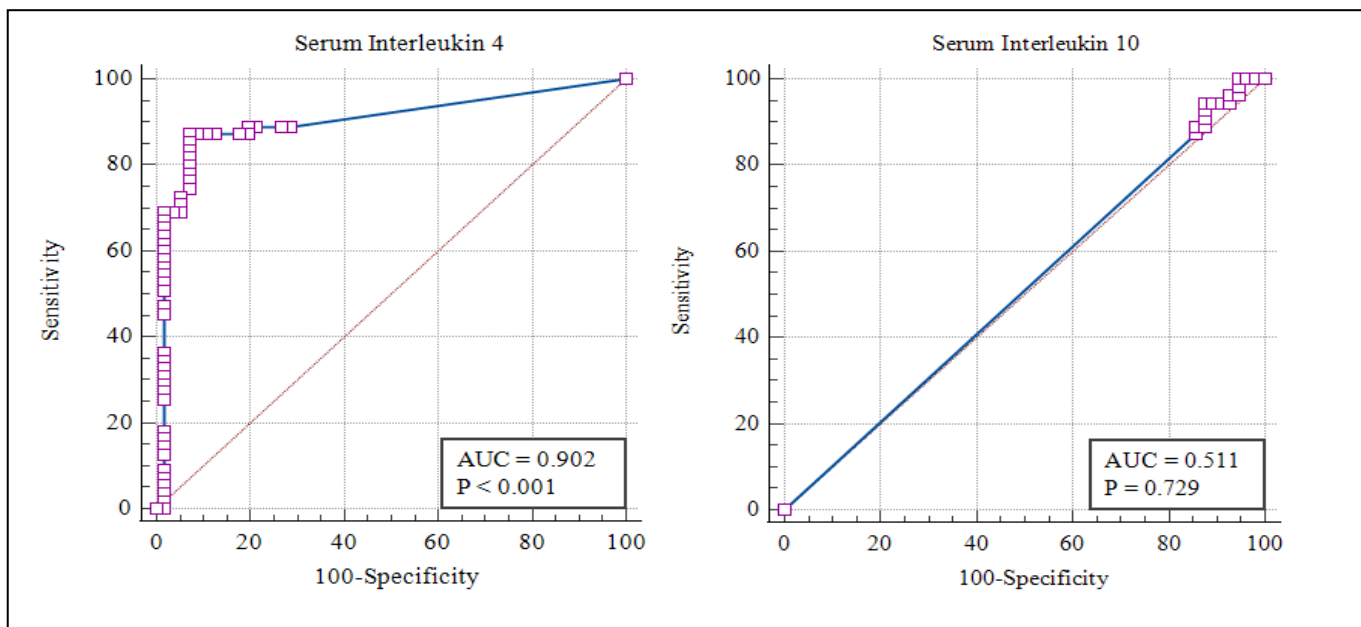
Table I: The demographic and clinical features of the participants.

Variables	Non-pregnant controls (n=32)	Pregnant controls (n=56)	Cases (n=55)	p-value
Age (years) ^a	26.72±5.35	25.77±5.67	28.33±5.93	.06
16-25 n (%)	16 (50.0)	34 (60.7)	18 (32.7)	.06
26-35 n (%)	13 (40.6)	18 (32.1)	31 (56.4)	
36-45 n (%)	3 (9.4)	4 (7.1)	6 (10.9)	
Gestational Age (weeks) ^a	N/A	30.20±1.70	30.82±1.82	.068
Blood Pressure (mmHg) ^a				
Systolic	105.94±9.46	105.71±9.5	147.82±16.85	<.001**
Diastolic	67.19±5.81	68.75±6.05	96.36±12.23	<.001**
Previous History of PE n (%)	0 (0.0)	0 (0.0)	10 (18.2)	<.001**
Proteinuria ^a	N/A	0±0	2.145 ± 0.95	<.001**

^aValues are presented as mean ± SD, and n (%) is the number of patients and frequency. N/A= Not applicable. *A p< 0.05 was considered significant. **A p< 0.001 was considered highly significant.

Table II: Levels of IL-4 and IL-10 in study participants.

Variables	Mean±SD	Median	IQR (25 th -75 th)	P value
IL-4 Levels (pg/ml)				
Whole study group (n=143)	39.82±41.59	21.0	7.79-54.90	<.001**
Non-pregnant controls (n=32)	110.02±23.8	107.9	95.1-119.1	
Pregnant controls (n=56)	10.20±9.27	7.79	7.79-9.6	
Early-onset Preeclampsia (n=55)	29.13±13.73 ⁺	32.20	16.00-38.4	
Intergroup comparison				
NPC vs PC				<.001**
PC vs EOP				<.001**
NPC vs EOP				<.001**
IL-10 Levels (pg/ml)				
Whole study group (n=143)	14.05±18.10	3.14	3.14-29.40	<.001**
Non-pregnant controls (n=32)	43.80±9.2 ⁺	43.65	39.53-47.95	
Pregnant controls	6.74±14.11	3.14	3.14-3.14	
Early-onset Preeclampsia (n=55)	4.19±4.37	3.14	3.14-3.14	
Intergroup comparison				
NPC vs PC				<.001**
PC vs EOP				.68
NPC vs EOP				<.001**

**Figure 2: ROC Curves of IL-4 and IL-10.****Table III: Cut-off values and values of validation criteria for IL-4 and IL-10 to differentiate between normotensive pregnancy and early-onset pre-eclampsia.**

Variables	IL-4	IL-10
Cut-off value	>11.6	≤3.14
Sensitivity %	87.27	87.27
Specificity %	92.86	14.29
Positive Likelihood Ratio %	12.22	1.02
Negative Likelihood Ratio %	0.14	0.89
Positive Predictive Value %	92.3	50.0
Negative Predictive Value %	88.1	53.3
Accuracy %	87.4	48.65

Discussion

In this study, significantly higher serum IL-4 levels were present in EOP as compared to pregnant controls. Because of the inflammatory etiology of preeclampsia, our discovery of elevated IL-4 levels in preeclamptic patients was unexpected. But similar results are derived from previous studies and our findings are comparable to their results. According to Omu *et al.*, serum levels of IL-4 were greater in the women with pre-eclampsia than their counterpart normotensive women throughout the pregnancy, and at the 24th week of gestation, their IL-4 levels were 39.0 versus 5.2

pg/ml with a $p < 0.05$.¹³ Mansouri *et al.*, also reported similar results: pre-eclamptic women had significantly increased levels of IL-4 (median: 22 and range: 0-185.2) as compared to normal pregnant women (median: 10.24 and range: 0-41.8) with a $p < 0.001$.¹⁴ It is appealing to think that raised IL-4 levels in EOP could be a compensatory strategy in response to elevated inflammatory cytokines.^{15,16}

Nevertheless, a few studies have shown conflicting results. Boij *et al.*, reported low IL-4 levels in early-onset preeclampsia (medians: 29; IQR: 18-57) as compared to normotensive females (median: 46; IQR: 31-83), with a p -value of 0.003.¹⁷ On the other hand, Cemgil *et al.*, reported that there was no association between serum IL-4 levels and PE, and levels of IL-4 were similar in women with healthy pregnancies and PE.¹⁸ According to a recent review by Bellos *et al.*, the role of IL-4 levels in PE is still contradictory, and further studies are needed to remove this inconsistency.¹⁹

Although non-pregnant controls had higher levels of interleukin-10 as compared to the cases and pregnant controls ($p < .001$), we found no significant change in IL-10 levels in EOP or normal pregnancy in this research. Our results are consistent with previous studies. Moreno-Eutimio *et al.*, detected no differences in the concentrations of plasma IL-10 at different stages of PE relative to normal pregnant subjects.²⁰ Similarly, Mansouri *et al.*, and Boij *et al.*, stated that the difference in IL-10 levels was statistically not significant in pregnant controls and pre-eclamptic females.^{14,17} Another study conducted by Dong *et al.*, revealed no significant differences in placental levels of IL-10 among women with normal pregnancy, gestational hypertension, or pre-eclampsia.²¹

In contrast, few studies have reported that preeclampsia is associated with lower IL-10 production. For example, in a case-control study of 56 preeclamptic and 50 normal pregnant females, preeclampsia was linked to reduced IL-10 production ($p < 0.05$).²² A similar investigation validated a substantial increase in IL-10 concentration in the serum of normotensive pregnant subjects compared to PE patients ($p = 0.01$).²³ Sharma *et al.*, also detected significantly lower IL-10 levels in pre-eclamptic subjects as compared to the pregnant controls.¹¹ A few studies reported higher IL-10 levels as well; for example, Ferguson *et al.*, detected high serum levels of IL-10 in the early trimesters of women who developed pre-eclampsia later in the third trimester.²⁴ Thus, levels in preeclampsia are still

conflicting to date, and these studies have concluded that serum IL-10 levels in preeclamptic patients have shown unpredictable results.

In our research, IL-4 at a cut-off value of >11.6 pg/ml had an excellent ability to differentiate the patients with EOP from normotensive pregnant females with a sensitivity of 87.27% and a specificity of 92.86%. This ROC curve analysis of IL-4 has never been done in previous studies and is a new contribution towards establishing serum IL-4 levels as a noninvasive biomarker for EOP. In the present study, the main limitation was the small sample size, and we collected samples from only one hospital using convenient sampling. Further studies including an expanded series of hospitals and a larger sample size are needed to remove this limitation.

Conclusion

In conclusion, EOP was associated with higher serum IL-4 levels as compared to pregnant controls. This research has contributed to a better understanding of the disease's complex etiology and could be a step toward establishing serum IL-4 levels as a noninvasive biomarker for EOP patients, which may not only help in early diagnosis but also in monitoring disease progression and therapy response. After establishing its role as biomarker, next step will be to explore the therapeutic potential of this cytokine in human subjects.

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