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SHORT COMMUNICATION

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Comparison of the percentages of $CD4^+$ $CD25^{high}$ $FOXP3^+$, $CD4^+$ $CD25^{low}$ $FOXP3^+$, and $CD4^+$ $FOXP3^+$ Tregs, in the umbilical cord blood of babies born to mothers with and without preeclampsia

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Problem: Little is known about how preeclampsia affects regulatory T-cell count and functions in umbilical cord blood of babies born to preeclamptic mothers. Here, we analyze the percentage of $CD4^+$ $CD25^{high}$ $FOXP3^+$, $CD4^+$ $CD25^{low}$ $FOXP3^+$, and $CD4^+$ $FOXP3^+$ Tregs, in the umbilical cord blood of babies born to mothers with and without preeclampsia.

Method of study: The percentage of umbilical cord blood $CD4^+$ $CD25^{high}$ $FOXP3^+$, $CD4^+$ $CD25^{low}$ $FOXP3^+$, and $CD4^+$ $FOXP3^+$ Tregs were analyzed by flow cytometry.

Results: $CD4^+$ $CD25^{high}$ $FOXP3^+$ Treg (%) and $CD4^+$ $FOXP3^+$ Treg (%) were significantly lower, while $CD4^+$ $CD25^{low}$ (%) was significantly higher in umbilical cord blood of babies born to preeclamptic mothers.

Conclusion: Preeclampsia is associated with immune dysregulation which leads to a deficiency in Treg ($CD4^+$ $CD25^{high}$ $FOXP3^+$) in the umbilical cord blood of babies born to preeclamptic mothers.

KEYWORDS
flow cytometry, FOXP3, preeclampsia, regulatory T cells, umbilical cord blood

1 | INTRODUCTION

Preeclampsia is a pregnancy-specific disorder characterized by a high blood pressure and a large quantity of protein in urine usually in the 3rd trimester of gestation.[1] It is a highly prevalent disorder as it affects 3%-17% of pregnancies worldwide and considered one of the important contributors to maternal and perinatal morbidity and mortality,[2] as it is responsible for 16% of maternal deaths in developed countries, while other causes like hemorrhage, abortion, and sepsis responsible for 13%, 8%, and 2%, respectively.[3] Although there are continuous research efforts trying to detect the exact etiology and pathogenesis of preeclampsia, it is still not completely understood, it is expected that an altered and persistent maternal systemic inflammatory response to pregnancy together with the endothelial damage caused by cytokine disturbance plays an essential role in the pathogenesis of PE.[4] Immunological incompatibility between the mother and her baby is due to differences in transplantation antigens inherited from the father as well as non-inherited maternal antigens.[5] So, adaptations in both the innate and adaptive immune system are needed to cope the semi-allogeneic conceptus and avoiding its rejection when it penetrates the endometrium and contacts maternal immune cells.[6]

Regulatory T cells (Treg) are variant populations of lymphocytes which regulate the adaptive immune response. $CD4^+$ Treg divides into natural (n Treg), which are thymus-derived $CD4^+$ $CD25^+$ $FOXP3^+$ T cells, and inducible (iTreg) (ie, Tr1 cells that secrete IL-10, Th3 cells that secrete TGF-β and IL-10).[7] Treg cells play important role in maintenance

of self-tolerance and immune homeostasis; the idea of expansion of Treg pool in the peripheral circulation of pregnant women and at the fetal-maternal interface is now well established.[8]

FoxP3 is an important marker for Treg development, its ectopic expression induces the suppressive function of Treg, it is expressed in most $CD4^+$ $CD25^+$ T cells and in a small part of $CD4^+$ $CD25^-$ T cells. CD25 is a useful cell surface marker for most of Treg cells.[9] Conventional Tregs can be classified into activated ($CD4^+$ $CD25^{high}$ $FoxP3^{high}$) and resting ($CD4^+$ $CD25^{high}$ $FoxP3^{low}$) Treg cells; each of them has the suppressive function but activated terminally differentiated Treg cells are more responsive and die rapidly, while resting Tregs have limited responsiveness, but can proliferate and convert into activated Treg cells with elevated FoxP3 expression.[10]

The exact role of Treg in normal pregnancy and unfavorable pregnancy outcomes is an exciting unresolved issue, especially their role in suppression of conceptus-specific conventional $CD4^+$ T cells that would otherwise attack fetal tissue.[11]

There are several studies that discussed Treg $FOXP3^+$ cells in uterine decidua[12,13] and in peripheral blood of preeclamptic women.[14-19] All studies which defined Treg by our same markers $CD4^+$ $CD25^{high}$ $FOXP3^+$ have reported the significantly lower level of Treg cells in uterine decidua and peripheral blood of preeclamptic patients than normal matched pregnant controls.

Regulatory T cells are involved in promoting fetal survival and in avoiding the recognition of paternal semi-allogeneic tissues by the maternal immune system. Many previous functional studies have found a strong association between unexplained infertility, miscarriage, and preeclampsia with the Treg cell number and function abnormalities,[20] but the effect of preeclampsia on fetal immune system is less well studied with limited studies regarding fetal Treg changes in preeclampsia

The purpose of this study was to compare the percentages of $CD4^+$ $CD25^{high}$ $FOXP3^+$, $CD4^+$ $CD25^{low}$ $FOXP3^+$, and $CD4^+$ $FOXP3^+$ Tregs, in the umbilical cord blood of babies born to mothers with and without preeclampsia.

2 | PATIENTS AND METHODS

A case-control study was conducted on fifty subjects. They were classified into two groups: Group (1) included thirty pregnant women with preeclampsia. Group (2) included twenty apparently healthy women with normal pregnancy who match the group (1) for age as a control.

Preeclampsia was diagnosed by obstetrics doctors, diagnosed when blood pressure is ≥140/90 mm Hg diastolic on 2 separate occasions, 4 hours apart after 20 weeks of gestation in a female with previously normal blood pressure, and proteinuria ≥300 mg/24 hours or ≥2 at dipstick urinalysis in absence of urinary tract infection; none of the patients with preeclampsia had preexisting clinical disorder.[21]

Patients were classified according to the onset of the disease into early-onset preeclampsia (>34 weeks of gestation) and late onset (≤34 weeks of gestation).[22] Another classification was done according to the degree of severity into mild (SBP 140-149 and DBP 90-99 mm Hg), moderate (SBP 150-159 and DBP 100-109 mm Hg), and severe preeclampsia (SBP ≥160 and DBP ≥110 mm Hg and/or with signs of renal, hepatic, or neurological impairment or thrombocytopenia).[23]

Exclusion criteria included twin pregnancies, fetal abnormalities, pregnancies complicated with clinical chorioamnionitis or any infectious disorder, patients receiving steroid or other immunosuppressive medicines, vaccines, patients with preexisting clinical disorders, such as diabetes, thrombophilia, chronic hypertension, or renal diseases before pregnancy and babies under 2000 g whether preterm or term.

2.1 | Consent and ethical committee

All patients gave written informed consent prior to being included in the study following a full explanation of the procedure. This study was approved by Benha faculty of medicine Research Ethics Committee (REC) at Benha University and conducted according to the principles of the Declaration of Helsinki.

2.2 | Sample collection

Umbilical cord blood was collected in a sterile cord blood collection bags (157 mL), after delivery of the baby by cesarean section, and the cord blood bag contained 22 mL of anticoagulant CPDA-1(citrate-phosphate-dextrose-adenine) which preserves red blood cells up to 35 days. It was collected by in utero technique before placental expulsion, from both groups.[24] We clamped the cord near the baby rather than the placenta. The cord blood unit was transported immediately to Mansoura Research Centre for Cord Stem Cells and was prepared for mononuclear cells separation.

2.3 | Sample processing

We checked the cord blood unit for total nucleated cells (TNC) which should be $\geq 5.0 \times 10^8$ TNC/unit according to International Standards for Cord Blood Collection, Banking, and Release of Foundation for the Accreditation of Cellular Therapy.

Mononuclear cell-layer separation was performed manually using density gradient centrifugation method by Ficoll-Paque media (Lymphoprep™; Fresenius Kabi Norge As, Halden, Norway). The steps of this method were done in laminar flow cabinet to reduce the risk of contamination. Cord blood was diluted 1:3 with RPMI 1640 medium (Stemcell Technologies, Vancouver, BC, Canada). A collection of buffy coat was done in clean 15-mL Falcon tube; then, equal amount of RPMI was added to it then centrifugation at 900 ***g*** for 10 minutes. Finally, we removed supernatant and added RPMI to 1 mL level.

After mononuclear cell separation, we assessed the viability of cells using either one of two methods. The first was by flow cytometry viability kit by adding 100 μL from mononuclear cells to 10 μL of viability dye and incubation for 20 minutes; then, we counted viability on flow cytometry. The second was manually by trypan blue stain by adding 10 μL of trypan blue stain to 10 μL of mononuclear cells and mixing

them together on a parafilm followed by application of 10 μL of the mixture to hemocytometer and counting viability by 10× microscope lens. The stained cells were dead and non-stained cells were viable.

The viability should be ≥85% viable nucleated cells according to International Standards for Cord Blood Collection, Banking, and Release of Foundation for the Accreditation of Cellular Therapy. Then, we counted mononuclear cells using automated cell counter in Mansoura University Children's Hospital (MUCH), before proceeding to an analysis by Multicolor Flowcytometry.

2.4 | Analysis by multicolor flowcytometry

The analysis was done using The BD Accuri™ C6 Plus personal flow cytometer. 100 000 events were recorded and analyzed by BD Accuri™ C6 Plus software. MNC were stained by human regulatory T-cell staining kit BD (eBioscience, San Diego, CA, USA, cat. 88-8995-40, lot. 4299752.), using anti-CD4 FITC, anti-CD25 PE cocktail and Rat IgG2a K Isotype Control PE-Cyanine5 and anti-human FOXP3 PE-Cyanine5.

2.5 | Statistical analysis

The data collected were tabulated and analyzed with the suitable statistical methods using SPSS program (IBM SPSS for Windows, version 20, Armonk, NY, USA). Categorical data were presented as number and percentage using chi-square (X^2) test to analyze them. Continuous variables were expressed as a mean ± standard deviation. Data were tested for normality using Shapiro-Wilk test, using Student "*t*" tests for normally distributed variables, or Kruskal-Wallis test, Mann-Whitney *U* (MWU) test, and Spearman's correlation coefficient (rho) for nonparametric variables. Significant Kruskal test was followed by post hoc multiple comparisons to detect the significant pairs using Bonferroni-adjusted Mann-Whitney *U* test at an adjusted *P* value of .005. For the comparison of the obstetric and maternal characteristics between the cases and controls, the Mann-Whitney *U* test and Fisher's exact test were used. A *P* value #.05 was considered statistically significant.

3 | RESULTS

Thirty preeclamptic pregnant females were enrolled in this study. Their mean age was 26.5 years. In addition to 20 age-matched apparently healthy women with normal pregnancy as a control group, preeclampsia group showed significantly lower GA; most of them had preterm labors ($P < .001$), as well as significantly lighter birth weighted infants ($P < .001$) than the control group. No significant differences were found regarding parity history between both groups. Mean PE onset was 30.9 weeks; 18 patients had an early onset (60%), while 12 patients had late onset (40%). Studied cases included mild, moderate, and severe cases (3, 21, and 6, respectively) (Table 1).

The frequency of $CD4^+$ $FOXP3^+$ (%) and $CD4^+$ $CD25^{high}$ $FOXP3^+$ (%) was significantly lower, while $CD4^+$ $CD25^{low}$ (%) was significantly higher in the cord blood of babies born to preeclamptic mothers when compared to cord blood samples from healthy babies born to healthy mothers. Mononuclear cells and lymphocytes did not differ significantly between both groups (Table 2).

Figure 1 shows an example for a preeclampsia case in which mononuclear cells were (8100 cells/μL), lymphocytes percentage of mononuclear cells was 73% (5913 cells/μL), $CD4^+$ cells percentage of lymphocytes was 42.8% (2530.7 cells/μL), other cell populations percentage of $CD4^+$ cells: $CD4^+$ $CD25^{low}$ was 43% (1088.2 cells/μL), $CD4^+$ $CD25^{high}$ was 8.3% (210.04 cells/μL), $CD4^+$ $FOXP3^+$ was 0.8% (20.24 cells/μL), and $CD4^+$ $CD25^{high}$ $FOXP3^+$ was 0.3% (7.59 cells/μL).

$CD4^+$ $FOXP3^+$ cells showed significant positive correlations with age, GA, $CD4^+$ $CD25^{high}$ $FOXP3^+$ cells in control group; GA, BW, $CD4^+$ $CD25^{high}$ $FOXP3^+$ cells in cases, and significant negative correlations with severity and $CD4^+$ $CD25^{low}$ cells in cases (Table 3).

$CD4^+$ $CD25^{low}$ cells (%) showed significant positive correlation with severity and significant negative correlations with age, GA, BW, onset, and $CD4^+$ $CD25^{high}$ $FOXP3^+$ in cases and showed significant positive correlation with age and GA in control (Table 4).

$CD4^+$ $CD25^{high}$ $FOXP3^+$ cells showed significant positive correlations with age and GA, in control group and with GA, BW, in cases, and significant negative correlation with severity in cases group (Table 5).

TABLE 1 Comparison between the cases and controls regarding obstetric and maternal characteristics

			Control N = 20		PE N = 30		*P*
Age (y)		Mean, SD	26.8	3.7	26.5	3.1	.758
Parity	Primipara	N, %	13	65	22	73.3	.529
	Multipara	N, %	7	35	8	26.7	
G. age (d)		Mean, SD	263.8	5.9	247	11	<.001
G. age	Preterm	N, %	6	30	22	73.3	<.001
	Term	N, %	14	70	8	26.7	
B. W. (g)		Median, range	3300	Range 2480-4300	2200	Rang 1300-3600	<.001
Onset (wk)		Mean, SD	-	-	30.90 ± 3.994		-

Age and GA are expressed as mean ± SD; compared by *t* test. Parity and GA categories are expressed as number and percentages, compared by chi-square and Fisher exact test. BW is expressed as median, range, compared by Mann-Whitney test.

TABLE 2 Comparison between the PE cases and controls regarding laboratory and flow cytometric characteristics (levels as percentages of $CD4^+$ cells)

	Control N = 20		PE N = 30		
	Median	Range	Median	Range	*P*
Mononuclear cells/μL	5650	3500-8100	6900	2600-8700	.352
Lymphocytes (%)	78.5	68-89	77	50-92	.526
$CD4^+$ $FOXP3^+$ (%)	3.7	1.5-6.8	1.45	0.4-4.3	<.001
$CD4^+$ $CD25^{low}$ (%)	5.2	2.5-11.9	29.9	4.9-56.1	<.001
$CD4^+$ $CD25^{high}$ $FOXP3^+$ (%)	2	0.8-3.5	0.8	0.2-2.2	<.001

Comparison done by Mann-Whitney test.

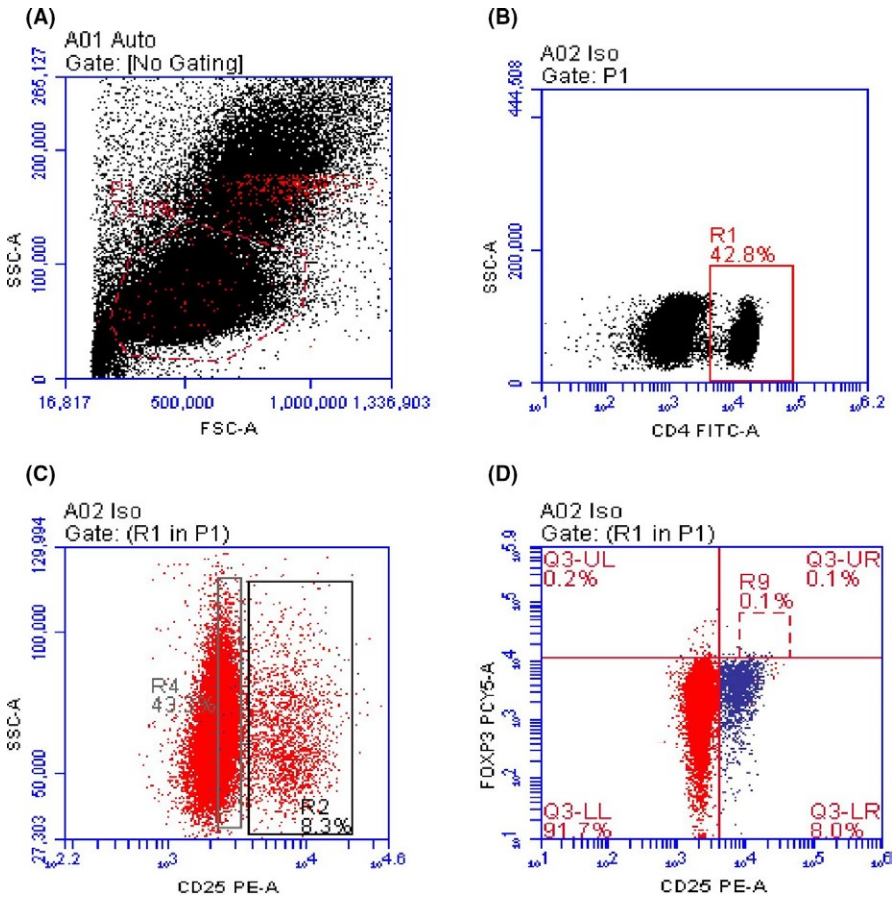

FIGURE 1 Preeclampsia case: (A) Gating of lymphocytes population. (B) Expression of CD4 positive cells in isotypic control and test on filter 1 (42.8%). (C) Expression of $CD4^+$ $CD25^{high}$ cells (8.3% on the right) and $CD4^+$ $CD25^{low}$ (43.0% on the left) in isotypic control and test on filter 2. (D) Negative expression of $CD4^+$ $CD25^{high}$ $FOXP3^+$ cells in isotypic control.

4 | DISCUSSION

Preeclampsia is accompanied by disturbances in the inflammatory status, together with activation of immune cells in both placenta and maternal circulation.[25] The present study compares the percentages of $CD4^+$ $CD25^{high}$ $FOXP3^+$, $CD4^+$ $CD25^{low}$, and $CD4^+$ $FOXP3^+$ Treg from $CD4^+$ cells, in the umbilical cord blood of babies born to preeclamptic mothers with cord blood samples from healthy babies born to healthy mothers.

The study revealed significantly lower percentages of $CD4^+$ $CD25^{high}$ $FOXP3^+$ and $CD4^+FOXP3^+$ Treg cells and significantly higher percentages of $CD4^+$ $CD25^{low}$ Treg cells in umbilical cord blood of babies born to preeclamptic patients, than in healthy control. This is paralleled with the hypothesis of the presence of immune dysregulation with defective regulatory T-cell distribution at the feto-maternal interface.[26]

The distribution of Treg cells in normal cord blood has not yet been defined. In several previous studies, the distribution of Treg cells in cord blood was reported as 2.63%-8.94%,[27] 4.0%-10.0%,[28] and 2%-3%.[29] However, these studies were limited as they enrolled small sample numbers. In 2012, an investigative study on the lymphocyte subsets in cord blood was undertaken for the first time. This study provided the reference intervals for lymphocyte subsets including Treg cells in umbilical cord blood from healthy full-term neonates; they conclude that the reference intervals for lymphocyte subsets were as follows: helper T cells ($CD3^+/CD4^+$), 15.40%-70.06%;

TABLE 3 Correlations between $CD4^+$ $FOXP3^+$ cells (%) and other studied parameters

	$CD4^+$ $FOXP3^+$ (%)			
	Control N = 20		PE N = 30	
	r	*P*	*r*	*P*
Age (y)	.514	.020	.233	.215
Gestational age (d)	.445	.049	.630	<.001
Birth weight (g)	.264	.261	.608	<.001
Onset (wk)	-	-	.158	.404
Severity	-	-	−.579	.001
Mononuclear cells/μL	−.099	.679	.037	.844
Lymphocytes (%)	.234	.322	−.171	.365
$CD4^+$ $CD25^{low}$ (%)	.030	.900	−.527	.003
$CD4^+$ $CD25^{high}$ $FOXP3^+$ (%)	.962	<.001	.943	<.001

Correlations done by Spearman's correlation.

TABLE 4 Correlations between $CD4^+$ $CD25^{low}$ cells (%) and other studied parameters

	$CD4^+$ $CD25^{low}$ (%)			
	Control N = 20		PE N = 30	
	r	*P*	*r*	*P*
Age (y)	−.026	.912	−.556	.001
Gestational age (d)	−.349	.132	−.525	.003
Birthweight (g)	.097	.684	−.428	.018
Onset (wk)	-	-	−.413	.023
Severity	-	-	.494	.006
Mononuclear cells/μL	.101	.672	.156	.410
Lymphocytes (%)	−.231	.328	.243	.195
$CD4^+$ $CD25^{high}$ $FOXP3^+$ (%)	−.044	.853	−.596	.001

Correlation done by Spearman's correlation.

TABLE 5 Correlations between $CD4^+$ $CD25^{high}$ $FOXP3^+$ cells (%) and other studied parameters

	$CD4^+$ $CD25^{high}$ $FOXP3^+$ (%)			
	Control N = 20		PE N = 30	
	r	*P*	*r*	*P*
Age (y)	.448	.048	.296	.112
Gestational age (d)	.490	.028	.774	<.001
Birth weight (g)	.163	.493	.691	<.001
Onset (wk)	-	-	.154	.416
Severity	-	-	−.675	<.001
Mononuclear cells/μL	−.102	.670	.127	.505
Lymphocytes (%)	.240	.308	−.217	.249

Correlation done by Spearman's correlation.

cytotoxic T cells ($CD3^+/CD8^+$), 9.65%-34.28%; B cells ($CD19^+$), 4.50%-29.59%; and natural killer cells ($CD3^-/CD16^+/CD56^+$), 1.42%-28.03%. The reference interval for Treg cells was 0.35%-9.07%, and this value was shown to have a wider range than the reference values (1.7%-7.0%) of adult peripheral blood.[30]

Our results are partially consistent with Prins et al;[31] they stated that only the frequency of $CD4^+$ $CD25^+$ T cells in umbilical cord blood showed statistically significant increases in preeclamptic female than normal pregnant females, while they did not find any difference in other Tregs stained positively with different antibodies.[31]

Confirming our talk about dysregulated cord blood Treg, another study was done by Loewendorf et al,[2] they compared between 3rd-trimester babies in preeclamptic and healthy pregnant females regarding the cord blood innate and adaptive immune cells phenotype. They found that the proportion of $CD4^+$ T cells was significantly decreased in the cord blood of babies born to preeclamptic mothers, with a significant reduction in the percentage of $FoxP3^+$ Treg, especially the FoxP3lo populations (resting Treg and cytokine Treg) in preeclamptic cord blood more than healthy controls.[2]

In this study, the severity of preeclampsia was negatively correlated with percentages of both $CD4^+$ $FOXP3^+$ and $CD4^+$ $CD25^{high}$ $FOXP3^+$ cells and positively correlated with the percentage of $CD4^+$ $CD25^{low}$ cells; also, the onset of preeclampsia was negatively correlated with a $CD4^+$ $CD25^{low}$ percentage.

$CD4^+$ $CD25^{high}$ $FOXP3^+$ is a population of $CD4^+$ cells expressing surface marker CD25 with an expression of regulatory intracellular transcription factor FOXP3. This phenotype is more conclusive for Treg cells, because $CD4^+$ $CD25^{high}$ cells may be shown with activation of conventional helper T cells. $CD4^+$ $FOXP3^+$ is a population of $CD4^+$ cells with expression of regulatory intracellular transcription factor FOXP3, Treg cells not only $CD25^+$ but also $CD25^-$ cells could exert regulatory function. $CD4^+$ $FOXP3^+$ cells have a broader view concerning the regulatory function of helper T cells.[13]

Preeclampsia is a multifactorial disease with many factors which contribute to making it manifested, in which a bidirectional effect may happen, and one is caused by defective numbers of Tregs at the feto-maternal interface because of immune dysregulation. This dysregulation occurs may be due to abnormal transplacental cytokine or hormonal expression (low IL-10, TGF-B or estrogens/high leptin or endoglin levels)[32,33] or defective transplacental transfer of chimeric cells (abnormal cell trafficking). This leads to shifting of immune system toward TH17 and TH1 direction with increased inflammatory cytokines and fetal rejection. The second effect of inflammation and negative feedback on Tregs leads to more defect in their numbers in cord blood with a vicious circle of inflammation.[34]

$CD4^+$ $CD25^{low}$ is a population of $CD4^+$ cells with increased CD25 expression indicating activation of helper T cells suggesting of provocation of the inflammatory process. We suggest that this population reflects the severity of inflammatory cascade that happens in preeclampsia. Thus, severe cases may be associated with higher percent of these cells.[35]

These results are consistent with Toldi et al[36] as they found that peripheral blood of preeclamptic females showed higher prevalence of activated T cells and exhausted Tregs in healthy pregnancy, and they conclude that the functionally most active effector Tregs was decreased in preeclampsia, while naive Tregs appear to be unaffected in PE compared with healthy pregnancy.[36]

While the results of Boij et al[37] did not find any difference in activated or resting Treg population between severe and early-onset preeclamptic females, and pregnant females without preeclampsia or non-pregnant females, but when they investigate functional and migratory Treg markers (CTLA-4 and CCR4), they concluded that the proportions of CTLA-4$^+$ and CCR4$^+$ were increased both in resting and inactivated Treg populations in untreated preeclamptic compared with normal pregnant females, and they decreased in normal pregnancy as compared with non-pregnant females, these differences were only in the non-corticosteroid treated group, and they suppose that this difference was due to the influence of corticosteroid treatment on the Treg phenotype.[37]

Another study done by Toldi et al[38] did not find significant differences in the frequency of CD4$^+$ CD25^{high} FoxP3$^+$ and CD4$^+$ CD25$^-$ FoxP3$^+$ cells, as well as in those of activated CD4$^+$ CD25^{high} FoxP3^{high} Tregs in PE patients, regarding early or late onset of PE (n of early PE = 11) or severity of PE (n of severe PE = 9).[38]

Different pattern of Tregs is observed by Hsu et al,[13] they suggest a systemic expansion of Tregs in healthy pregnancy through induction of CD4$^+$ Helios$^-$ FoxP3$^+$ adaptive Treg (iTreg) cells and not CD4$^+$ Helios$^+$ FoxP3$^+$ (nTreg) cells and conclude that this ratio is reversed in decidua of preeclamptic women.[13]

PE is associated with marked dysregulations in Tregs as it is associated with decreased CD4$^+$ CD25$^+$ FoxP3^{high} Treg cells and increased CD4$^+$ CD25^{high} FoxP3$^+$ Treg cells,[39] these findings mean that effector Treg cells are decreased, and effector T cells are increased in PE.

5 | CONCLUSION

This study concludes that preeclampsia is associated with immune dysregulation leads to deficiency in Treg (CD4$^+$ CD25^{high} FOXP3$^+$) count at feto-maternal interface represented in umbilical cord blood of babies born to preeclamptic mothers when compared to cord blood samples from healthy babies born to healthy mothers.

6 | RECOMMENDATIONS

Further studies on larger number of samples, and using other Treg-associated markers as CTLA4, are recommended.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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