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ORIGINAL ARTICLE

# Levels of Co-inhibitory Receptors TIM- 3, LAG-3 and TIGIT in Preeclampsia Patients

**[1]Zeinab I. Sayed*, [1]Noha A. Afifi, [1]Nahla M. Elsherbiny, [2]Reda S. Hussein, [1]Asmaa S. Shaltout**

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University
[2]Department of Obstetrics and Gynecology, Faculty of Medicine, Assiut University

***Key words:***
***Preeclampsia, co-inhibitory receptor, TIM- 3, LAG-3 and TIGIT***

***Corresponding Author:***
Zeinab Ibraheem Sayed Abdel Fadeel
Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University
Tel: 01024285328
zeinababdelfadeel@med.aun.edu.eg

## ABSTRACT

***Background:*** *Preeclampsia (PE) is a hypertensive disorder that affects 2-15% of pregnancies and is linked to immune system overactivation. Co-inhibitory receptors play a key role in regulating the immune system during pregnancy.* ***Objective:*** *This study aimed to compare expression levels of co-inhibitory receptors LAG-3, TIM-3 and TIGIT in blood and decidual tissue, as well as the serum IL10 levels between preeclampsia patients and those with normal pregnancy and to assess their diagnostic potential in predicting preeclampsia including those with severe features.* ***Methodology:*** *Blood and decidual tissue samples were collected from 24 patients with PE and 24 healthy pregnant controls. Expression of TIM-3, TIGIT and LAG-3 genes was determined by RT-qPCR. Serum IL-10 levels were measured by ELISA in both groups.* ***Results:*** *our results showed significantly reduced expression in blood TIM-3, LAG-3 and TIGIT as well as decidual LAG-3 and TIGIT among PE patients compared to controls. The decrease in expression levels was significantly pronounced in the blood than in the decidua of PE patients. Serum levels of regulatory cytokine IL10 were also significantly lower in PE cases compared to controls, with a significant positive correlation with tissue TIM-3. Additionally, TIGIT expression was significantly lower in PE patients with severe features compared to those without.* ***Conclusions:*** *The expression levels of immune checkpoints (TIM-3, LAG-3, and TIGIT) and IL-10 in the blood and decidua of patients with preeclampsia (PE) differ from those in healthy pregnancies, indicating their potential role in the pathogenesis and prediction of PE and its complications.*

## INTRODUCTION

Pre-eclampsia (PE) is a pregnancy related complication affecting maternal and fetal health, with a global prevalence of 2 to 15% [1]. Maternal tolerance to paternal antigens of the fetus is crucial for a successful pregnancy [2]. Early detection and understanding of PE's causes are essential, as the primary treatment involves premature delivery [3].

Preeclampsia is a condition marked by newly increased blood pressure (≥ 140/90) after twenty weeks' gestation, along with proteinuria, renal insufficiency, thrombocytopenia, impaired liver function, or new-onset headache or visual disturbances. PE with severe features is defined as severe range hypertension (SBP ≥160, or DBP ≥110 mm Hg, or both) with at least one of the following:(eclampsia, HELLP syndrome, intra uterine growth restriction, and pulmonary oedema[4].

The pathogenesis and etiology of PE are likely to be multifactorial and still unclear. Inappropriate placentation and insufficient trophoblast invasion are the main theories, leading to the release of antiangiogenic factors and inflammatory mediators [5]. Also, altered genetic changes, disrupted gut bacteria and imbalanced blood vessel growth factors may contribute to its development [6].

For a normal successful pregnancy to occur, the maternal immune system must tolerate the semi-allogeneic fetus by different mechanisms. CD4+, CD8+, and Treg play a crucial role in maintaining pregnancies[7]. Balance between Th1/Th2/ Th17 is essential for a successful pregnancy. Thus, in normal pregnancy there is an increase in Th2 and Treg subsets, along with a with decrease in Th1 and Th17 cells as well as pro-inflammatory cytokines [8]. Ultimately, dysregulation of Th cell immunity during pregnancy may result in PE [9].

Immune checkpoint molecules, such as T cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell immunoglobulin and ITIM domain (TIGIT), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD-1), and Lymphocyte activation gene-3 (LAG-3), play a crucial role in balancing pro-inflammatory and anti-inflammatory signals in the immune system [10 ; 11]. These molecules are expressed on activated T cells and help regulate the function of effector T cells and promote the suppressive function of regulatory T cells, thereby maintaining immune homeostasis [12].

Various world-wide studies have presented conflicting findings regarding the potential role of co-inhibitory receptors in PE. Some studies have reported lower levels of these receptors in PE compared to normal pregnancy [7,12,13], while others have found no abnormal expression of immune checkpoint molecules in PE patients [3]. Additionally, TIM-3 was even found to be upregulated in decidua of preeclamptic women in another study [14].

Moreover, the use of these receptors as predictive markers for PE and its complications has not been established. To address this gap in knowledge, we conducted this study to investigate the expression levels and predictive values of co-inhibitory receptors (in the blood and decidual tissue) and IL10 levels in Egyptian women with PE, including those with severe features, compared to normal pregnancy.

## METHODOLOGY

This cross-sectional study was approved by the Institutional Ethical Review Board of the Faculty of Medicine at Assiut University, Egypt (IRB no 17200703) and registered on Clinical Trials.gov ID: (NCT05294952). Written consents were obtained from the participants in the study. The Sample size was calculated based on previous studies (15), witha power of 85% (using one sided test and α of 0.05), requiring a total of 48 participants (24 participants in each group)

The study included 48 pregnant women recruited between October 2022 and October 2023, divided into two groups; Group I: comprised twenty-four pregnant women >20 weeks gestational age diagnosed with PE or PE with sever features according to ACOG criteria4. Exclusion criteria included multifetal gestation, autoimmune diseases, acute inflammatory diseases, chronic diseases, and gestational diabetes mellitus or pre-existing diabetes mellitus. **Group II:** consisted of twenty-four normotensive pregnant women without proteinuria serving as the control group.

***Sample collection***

Four ml venous blood were drawn from PE patients and healthy pregnant women. Two ml were collected in serum tubes, and the serum was stored at -20 °C for the enzyme-linked immune-sorbent assay (ELISA). The remaining two ml were collected in tubes containing EDITA for total RNA extraction. Decidual tissues were collected after delivery and dissected from the maternal surface of the placenta. The tissues were sliced, transferred to 700 ul of RNA lysis buffer, and stored in Eppendorf tubes at -80°C till further use.

***RNA extraction and real-time PCR***

Total RNA was extracted from blood and decidual tissue according to the manufacturer's instructions (Applied Biotechnology, USA cat. number: ABT002). Complementary DNA (cDNA) was synthesized from RNA using Revert Aid Reverse Transcriptase kit (Applied Biotechnology 2X RT Mix, USA). The house keeping gene B-actin was used for normalization of data and triplicate readings were recorded. Gene-specific primers and SYBR Green Master Mix (DBI, Nuremberg, Germany) were used in the real-time PCR system (Roche Applied Science, Germany). For B-actin, the PCR conditions were initial denaturation at 95 °C for 10 min (1 cycle), followed by denaturation at 95 °C for 1 min, and annealing at 60 °C for 30 s (45 cycles) [16]. For TIGIT, TIM3 and LAG3), the PCR conditions were initial denaturation at 95°C for 3 min (1 cycle), followed by denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72°C for 30 s (40 cycles) [17]. The used primer sequences are shown Table 1.

**Table 1: The primers used in RT- qPCR [16,17]**

| Genes | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|
| TIM-3 | TCCAAGGATGCTTACCACCAG | GCCAATGTGGATATTTGTGTTAGATT |
| LAG-3 | GCGGGGACTTCTCGCTATG | GGCTCTGAGAGATCCTGGGG |
| TIGIT | TCTGCATCTATCACACCTACCC | CCACCACGATGACTGCTGT |
| β-actin | AGAGCTACGAGCTGCCTGAC | AGCACTGTGTTGGCGTACAG |

***Enzyme -linked immunosorbent assay for serum IL-10***

Serum levels of IL-10 in patients and controls were measured using ELISA kits (ELK Biotechnology, USA, Cat: ELK1142) following the manufacturer's protocol. The assay is based on the double-antibody sandwich technique, and duplicate readings were recorded.

**Statistical analysis:**

Statistical analyses were conducted using statistical package for the social science (SPSS), version 22. Data were statistically described in terms of mean ± standard deviation (± SD), median, range, frequencies (number of cases) and relative frequencies (percentages). Comparison of quantitative variables was done using Student's t-test (for normally distributed data) and Mann-Whitney U test (for non-normally distributed data). Wilcoxon rank test was used for comparing paired quantitative data. Categorical data were compared using the Chi-square test or Fisher's exact test when the expected frequency was less than five. Pearson correlation test was used to assess the relationship between variables. Receiver Operating Characteristic Curve (ROC) analysis was performed to determine optimal cut-off values for predicting

preeclampsia. A significance level of 0.05 was used for all analyses [18].

## RESULTS

The clinical and demographic data of patients included in this study are presented in Table 2.

The median systolic and diastolic blood pressure was significantly higher among the studied cases with PE compared to matched controls ($p$<0.001). All studied cases were positive for albumin in urine versus controls were negative ($p$ <0.001), additionally seven cases (29.2%) had a history of previous preeclampsia versus no controls had previous preeclampsia ($p$ =0.009).

Among the studied 24 preeclampsia cases; eclampsia was the commonest complication as it was documented in eight cases (33.3%), followed by HELLP syndrome in four cases (16.7%), pulmonary oedema in one case (4.2%), and intrauterine growth retardation in another one case (4.2%).

***Blood and tissue expression levels of co-inhibitory-receptors TIM-3, LAG-3 and TIGIT in preeclampsia cases and controls.***

Median LAG-3 and TIGIT expression levels were significantly lower in both blood and tissues of PE cases compared to controls. Blood TIM-3 expression level was also significantly lower in PE cases with no decrease in tissue levels (Figure 1).

***Expression levels of co-inhibitory-receptors TIM-3, LAG-3 and TIGIT in blood versus tissue in preeclampsia cases***

The expression levels of TIM-3, LAG-3 and TIGIT in blood were significantly lower than in tissues (**Table 3).**

***Serum IL-10 levels in preeclampsia cases and controls***

Serum IL-10 levels were significantly lower in the PE cases compared to controls (Figure 2).

***Correlation between serum IL-10, and co-inhibitory-receptors in preeclampsia patients***

A significant positive correlation was found between TIM-3 tissue expression levels and serum IL-10 ($r$=0.458, $p$=0.025). Additionally, a significant positive correlation was observed between tissue expression of TIM-3 and LAG-3 ($r$=0.405, $p$=0.050). Other correlations were not statistically significant as shown Figure 3.

***ROC curve analysis for evaluation of IL10 and co-inhibitory receptors as markers for PE and PE with severe features***

Serum IL10 and levels of co-inhibitory receptors in blood and tissues, except for tissue TIM-3, were found to be significant predictors of PE. Among these, tissue TIGIT showed 100% accuracy in predicting PE as illustrated in Figure 4 and Table 4. In addition, tissue TIGIT showed 70.8% accuracy in predicting PE with severe features (Table 5).

**Table 2: Demographic and clinical data of the studied participants**

| Demographic data | Preeclampsia (n=24) | | Controls (n=24) | | P value |
|---|---|---|---|---|---|
| **Age (years)** | | | | | 0.801 |
| • Mean ± SD | 27.12 ± 5.19 | | 26.75 ± 5.08 | | |
| • Median (range) | 28 (18 – 35) | | 28 (19 – 35) | | |
| **SBP, median (range)** | 155 (140 – 170) | | 120 (120 – 120) | | **<0.001** |
| **DBP, median (range)** | 100 (90 – 110) | | 80 (80 – 80) | | **<0.001** |
| **Parity, n (%)** | | | | | 1 |
| • Nulliparous | 11 | (45.8) | 11 | (45.8) | |
| • Multipara | 13 | (54.2) | 13 | (54.2) | |
| **Albumin in urine, n (%)** | | | | | **<0.001** |
| • None | 0 | (0.0) | 24 | (100.0) | |
| • +2 | 2 | (8.3) | 0 | (0.0) | |
| • +3 | 9 | (37.5) | 0 | (0.0) | |
| • +4 | 13 | (54.2) | 0 | (0.0) | |
| **Previous preeclampsia, n (%)** | | | | | **0.009** |
| • No | 17 | (70.8) | 24 | (100.0) | |
| • Yes | 7 | (29.2) | 0 | (0.0) | |
| **Developed complication, n (%)** | | | | | |
| • Eclampsia | 8 | (33.3) | | | |
| • HELLP | 4 | (16.7) | | | |
| • Pulmonary edema | 1 | (4.2) | | | |
| • IUGR | 1 | (4.2) | | | |

DBP: diastolic blood pressure. HELLP: Hemolysis, Elevated Liver enzymes, Low Platelet count.
IUGR: intrauterine growth restriction. SBP: systolic blood pressure
*Significance defined by $p$ < 0.05.
# Two cases had both eclampsia and HELLP

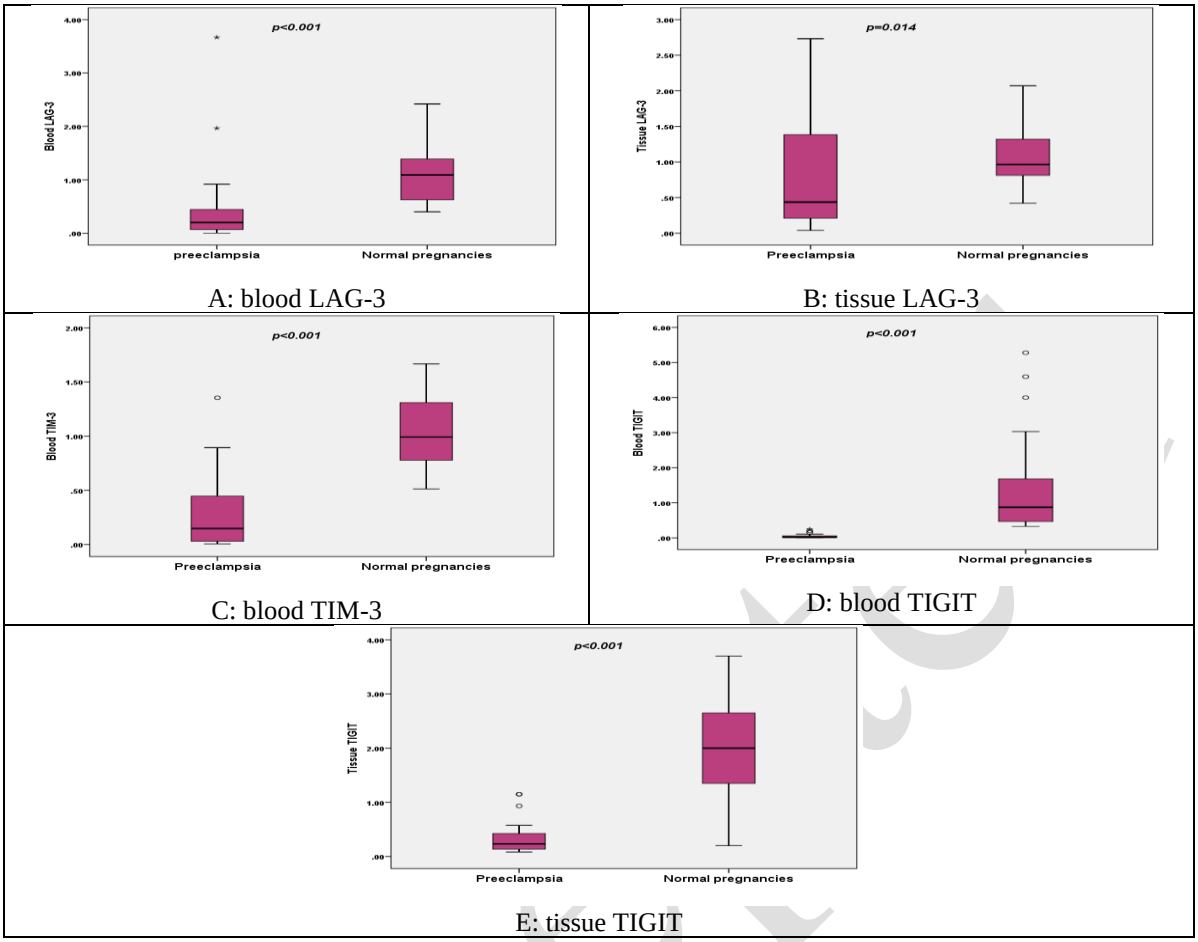


**Fig. 1:** Expression levels of co inhibitory receptors in PE and normal pregnancy

**Table 3: Expression levels of co-inhibitory-receptors TIM-3, LAG-3 and TIGIT in blood versus tissue in preeclampsia patients**

| Genes | Tissue | Blood | *p* value |
|---|---|---|---|
| **LAG-3** | | | |
| Median (range) | 0.44 (0.04 - 2.73) | 0.20 (0.002 - 3.67) | **0.022*** |
| **TIM-3** | | | |
| Median (range) | 0.68 (0.04 - 2.35) | 0.15 (0.01 - 1.35) | **0.022*** |
| **TIGIT** | | | |
| Median (range) | 0.23 (0.08 - 1.15) | 0.02 (0.002 - 0.25) | **<0.001*** |

*Significant *P* value <0.05

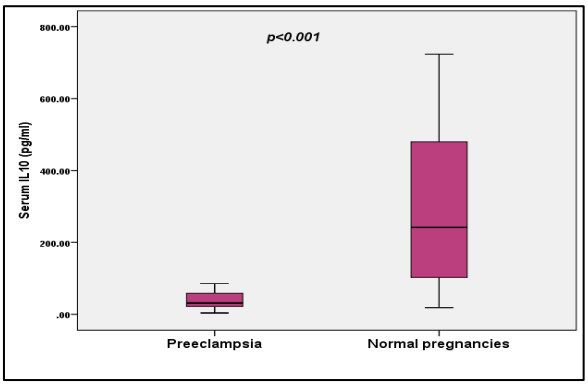


**Fig. 2:** Serum IL10 levels between preeclampsia cases and controls.

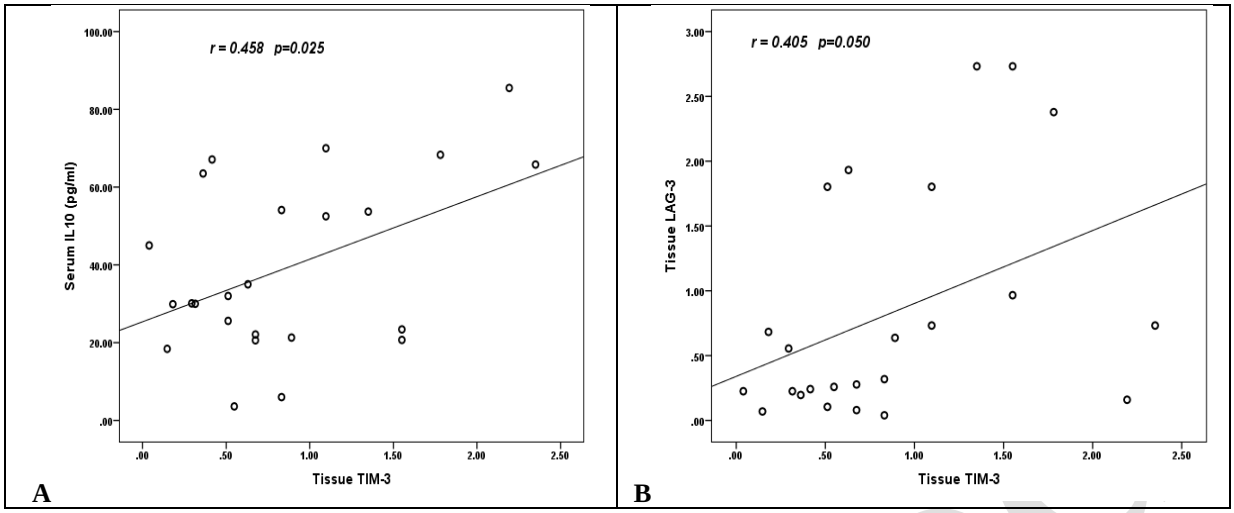


**Fig. 3:** Correlation between: (A) tissue TIM-3 expression and serum IL10, (B) tissue TIM-3 and tissue LAG-3 expression levels among the preeclampsia patients.

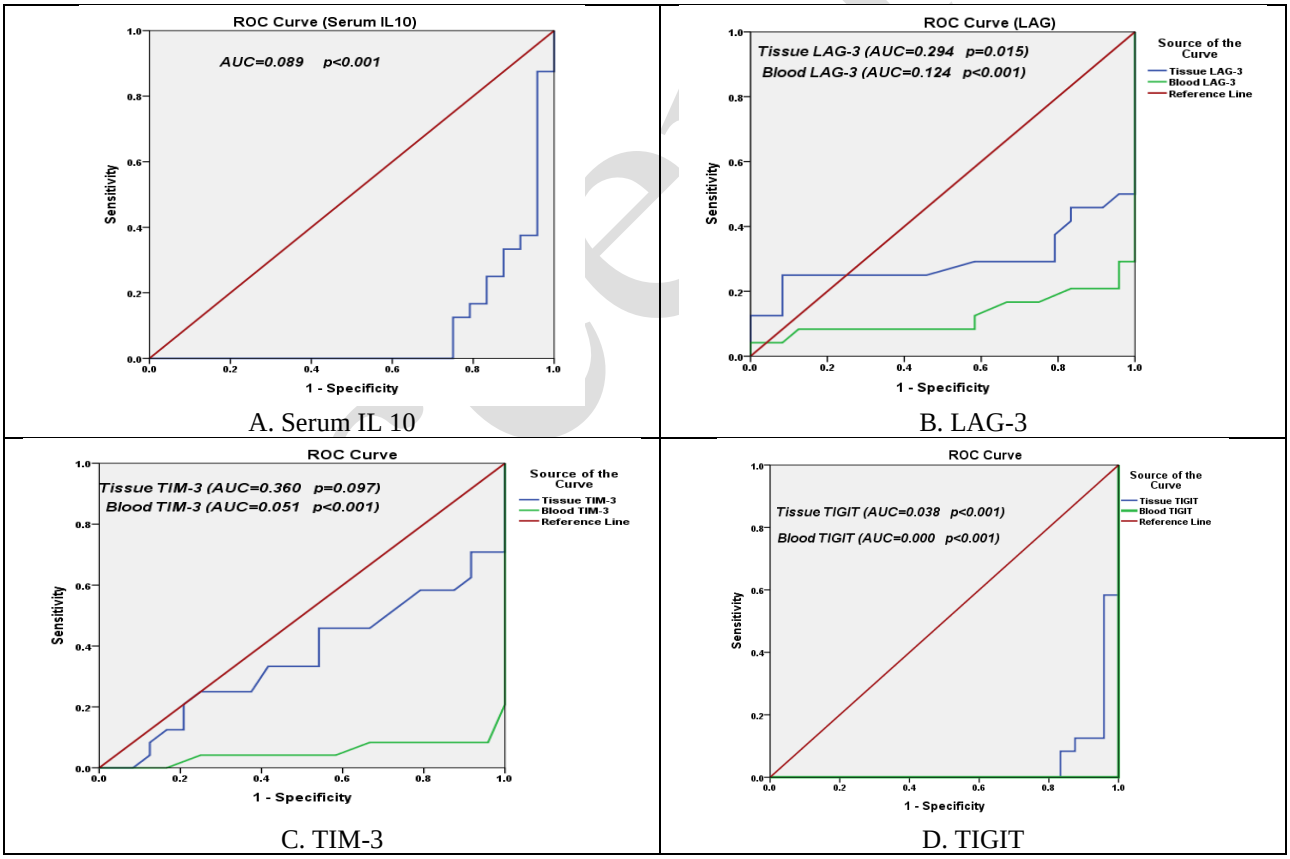


**Fig.4:** ROC curves for prediction of preeclampsia

**Table 4: Diagnostic accuracy of serum IL 10 and co- inhibitory receptors expression levels for prediction of preeclampsia**

| Markers | Cut off | 95%CI | Sensitivity | Specificity | PPV | NPV | Accuracy | AUC | *p*-value |
|---|---|---|---|---|---|---|---|---|---|
| **Tissue TIM-3** | ≤ 0.70 | 0.201–0.519 | 54.2% | 66.7% | 61.9% | 59.3% | 60.4% | 0.360 | 0.097 |
| **Blood TIM-3** | ≤ 0.53 | 0.00–0.122 | 91.7% | 95.8% | 95.7% | 92.0% | 93.8% | 0.051 | **<0.001** |
| **Tissue LAG-3** | ≤ 0.76 | 0.129–0.459 | 70.8% | 79.2% | 77.3% | 73.1% | 75.0% | 0.294 | **0.015** |
| **Blood LAG-3** | ≤ 0.49 | 0.011–0.237 | 79.2% | 95.8% | 95.0% | 82.1% | 87.5% | 0.124 | **<0.001** |
| **Tissue TIGIT** | ≤ 0.64 | 0.00–0.091 | 87.5% | 95.8% | 95.5% | 88.5% | 91.7% | 0.038 | **<0.001** |
| **Blood TIGIT** | ≤ 0.29 | 0.00–0.00 | 100.0% | 100.0% | 100.0% | 100.0% | 100.0% | 0.00 | **<0.001** |
| **Serum IL10 (pg/ml)** | ≤ 66.0 | 0.002–0.175 | 83.3% | 79.2% | 80.0% | 82.6% | 81.3% | 0.089 | **<0.001** |

PPV: positive predictive value; NPV: negative predictive value; AUC: Area under the curve; CI: confidence interval.
*Significance defined by $p < 0.05$

**Table 5: The diagnostic accuracy of tissue TIGIT expression levels for prediction of complicated preeclampsia**

| Marker | Cut off | 95%CI | Sensitivity | Specificity | PPV | NPV | Accuracy | AUC | *p*-value |
|---|---|---|---|---|---|---|---|---|---|
| **Tissue TIGIT** | ≤ 0.22 | 0.040 – 0.418 | 66.7% | 75.0% | 72.7% | 69.2% | 70.8% | 0.229 | **0.024** |

PPV: positive predictive value; NPV: negative predictive value; AUC: Area under the curve; CI: confidence interval.
*Significance defined by $p < 0.05$

## DISCUSSION

Preeclampsia is a serious pregnancy-related complication, with increasing incidence in developing countries[1]. Without proper healthcare, it can result in preterm birth and fetal growth restriction [19]. The exact cause of PE is still unknown, but it is believed that maternal inflammation restricts the migration of the cytotrophoblasts, leading to endothelial injury and inadequate implantation resulting in a small-sized placenta [5].

This study examined the expression levels and predictive values of co-inhibitory receptors (TIM- 3, LAG-3 and TIGIT) and IL10 levels in Egyptian women with PE, including those with severe features, compared to normal pregnancy. The results indicated that blood LAG-3 expression levels were significantly lower in patients with PE compared to those with normal pregnancy. This finding is consistent with previous studies that have shown reduced LAG-3 expression on various immune cells in PE compared to normal pregnancy[20,12]. We also found that decidual LAG-3 expression was significantly reduced in PE patients compared to normal pregnancy, which is in agreement with the study of Madadi *et al.*[13].

LAG-3 and CD4 have similar structures, but LAG-3 binds to MHC-II molecules more strongly than CD4. The interaction between LAG-3 and MHC-II negatively regulates T cell activation. During normal pregnancy, T cells express high levels of LAG-3 to suppress T cell effector responses, playing a crucial role in maintaining normal pregnancy through various inhibitory mechanisms[21]. Reduced expression of LAG-3 in preeclampsia leads to abnormal T cell activation and contributes to impaired maternal immune tolerance [20].

Regarding blood TIM-3, our study again revealed a significantly reduced expression in PE patients compared to normal pregnancies, consistent with the previous findings of Miko *et al.* [22] and Wang *et al.* [9], who concluded that the altered TIM-3/Gal-9 system may increase the pro-inflammatory Th1 response resulting in enhanced systemic inflammatory response in PE.

Conversely, some studies have reported an upregulation of TIM-3 in PE patients, suggesting failure of regulating Th1 and Th17 thus producing a hyperimmune response that affects spiral artery remodeling leading to immune rejection of the fetus [14]. Thus, the role of TIM-3/Gal-9 in the pathophysiology of PE is currently under investigation. It serves as a crucial immunoregulatory molecule that can suppress Th1 immunity and on the other hand, promote inflammation[23]. TIM-3 is expressed on various immune cells and can have stimulatory or inhibitory effects on the immune system depending on the cell type [24].

This study supports the perspective that TIM-3 and its ligand, Gal-9, are immune checkpoint proteins crucial for balancing proinflammatory and anti-inflammatory signals mediated by T cells. The TIM-3/Gal-9 pathway promotes maternal-fetal tolerance by inducing regulatory T cells and Th2 cells, leading to an anti-inflammatory effect on the immune system [25]. Thus, we reported a significant positive correlation between the level of TIM-3 in the decidua and each of serum IL 10 and decidual LAG-3 level which agrees with previous studies[9,13]. This supports the fact that both co-inhibitory receptors and IL10 play a key role in regulating T cell responses and maintaining immune homeostasis during pregnancy[13].

Although TIM-3 expression in blood was significantly lower than in the decidua in PE, yet there was no significant difference in TIM-3 levels in the decidua between (PE) and normal pregnancy. This aligns with the study of Powell *et al.* [26], who reported that TIM-3 supports the maintenance and survival of CD8+ T-cell subset at the maternal-fetal interface.

Our study also found that blood TIGIT expression levels were significantly lower in PE patients compared to normal pregnancy, consistent with previous studies that also reported decreased TIGIT expression on different immune cell subsets in PE cases [7,27]. However, Li *et al.* [3] didn't find a significant difference in TIGIT and CD155 levels between PE and normal pregnancy. We also found that TIGIT levels in the decidua of patients with PE were significantly lower than in normal pregnancies. This is the first reported data on TIGIT expression in the decidua in PE.

However, Fu *et al.*[28] reported that TIGIT expression in the decidua of early normal pregnancy was significantly higher than that of unexplained miscarriage. The reduced TIGIT expression in PE may lead to a Th1 bias, potentially exacerbating the development of the condition. The TIGIT/CD155 pathway promotes healthy pregnancy by inhibiting IL-12 production from APCs and inducing Treg cell and IL-10 production, shifting the balance from Th1 to Th2 immunity [7]. This explains the relatively high accuracy of tissue TIGIT in predicting severe PE in this study.

The significantly lower serum IL10 concentrations observed in PE cases compared to normal pregnancy is in accordance with many recent studies[7,29,30]. The exacerbation of the maternal inflammatory response, marked by increased production of IL-1β, and TNF-α and decreased concentrations of IL-10 and TGF-β is the main inducer of PE[8].

## CONCLUSIONS

The expression levels of immune checkpoints (TIM-3, LAG-3, and TIGIT) and IL-10 in the blood and decidua of patients with preeclampsia (PE) differ from those in healthy pregnancies, indicating their potential role in the pathogenesis and prediction of PE and its complications.

In our study, we found that most of the studied co-inhibitory receptors, except tissue TIM-3, could serve as reliable biomarkers for diagnosing PE, showing considerable sensitivities and specificities. Blood TIGIT was the most accurate, with 100% sensitivity and specificity. The study was limited by its small sample size, focusing primarily on patients at around 36 weeks' gestation at delivery, and not analyzing the expression and roles of these molecules on various immune cells.

**Conflict of interest**

The authors declare that they have no competing interests.

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**Abbreviations:**

**IL:** Interleukin; **LAG-3:** Lymphocyte activation gene-3 PE: preeclampsia; **TIGIT:** T cell immunoglobulin and ITIM domain; **Tim-3:** T-cell immunoglobulin domain and mucin domain molecule 3

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