

# Differences in the Expression of TLRs and Inflammatory Cytokines in Pre-Eclamptic Compared with Healthy Pregnant Women

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## ABSTRACT

**Background:** Preeclampsia (PE) is one of the most complex and life-threatening pregnancy disorders and is considered as a major cause of mortality among mothers and fetuses worldwide. Although the exact etiology of PE is not well known several lines of evidence support an immunological etiology for PE. **Objective:** To investigate the differences in the expression of TLRs 2, 4, 5, and 6 and a group of inflammatory cytokines including IL-1, IL-6, TNF- $\alpha$  and IFN- $\gamma$  in placentas from PE and healthy pregnant women in their third trimester of pregnancy. **Methods:** This case-control study was performed on fifteen PE and fifteen age and gestational matched healthy pregnant women in the third trimester of pregnancy. Real time PCR (RT-PCR) technique was used to determine the expression of TLRs 2, 4, 5, and 6 in the maternal and fetal parts of the placenta. Moreover, the expressions of IL-1, IL-6, TNF- $\alpha$  and IFN- $\gamma$  at RNA level in placental samples, peripheral, and cord blood were investigated. **Results:** The results of the present study indicated that the expressions of TLRs 4, 5 and 6 were significantly increased in both maternal part ( $p < 0.001$  and  $p < 0.003$  for TLRs 4, 6 and TLR 5, respectively) and fetal part ( $p < 0.001$ ), while TLR2 showed significant increase only in the fetal part of PE placentas ( $p < 0.002$ ). The levels of all studied cytokines showed over-expression within peripheral and cord blood samples from PE patients ( $p < 0.001$  for IL-1, IL-6, and IFN- $\gamma$  and  $p < 0.004$  for TNF- $\alpha$  in both cord and peripheral blood samples). **Conclusion:** The finding of the present study indicated that the expression of the studied TLRs and inflammatory cytokines are generally suppressed in normal pregnancy, but are up regulated in preeclamptic women. Moreover, it seems that the maternal and fetal parts of the placenta may play different roles in the induction of the inflammatory status within the placenta.

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## INTRODUCTION

Preeclampsia (PE) is one of the most complex pregnancy disorders with an unknown etiology. PE occurs in up to 8% of all pregnancies worldwide and presents with maternal hypertension and proteinuria after 20 weeks of gestation (1). PE accounts for up to 18.2% of maternal deaths and 12% of preterm deliveries in Iran (2). The most common maternal manifestations of PE are new-onset of hypertension (blood pressure >140/90 mmHg), occurring after the 20<sup>th</sup> week of gestation, which normalizes after delivery, and proteinuria (> 0.3g/ 24h) (3). The levels of blood pressure and proteinuria further determine the severity of the disease (4). Although the exact etiological factors of PE are not well known, there is no doubt that it is a placental disease (5). Indeed, a defect in placental formation might lead to PE (6). The innate immune system is essential for a normal implantation and placentation. During the pregnancy period, the innate immune system at the maternal-fetal interface actively works to form a normal placentation, protect the placenta against the invasion of microorganisms, and tolerate the semi-allograft fetus (7,8).

On the other hand, cytokines play several important roles from fertilization up to delivery. Cytokines are also important immune factors that regulate immune responses and communications between the fetus and mother (9).

Toll-Like Receptors (TLR) are the best known family of Pattern Recognition Receptors (PRRs) that are involved in innate immunity. TLRs recognize Pathogen or Danger Associated Molecular Patterns (PAMPs and DAMPs). In addition to the cells of the innate immune system, non-immune cells such as epithelial and trophoblast cells also express TLRs that allow these cells to respond to PAMPs and DAMPs by producing inflammatory cytokines (10). Although the expression of all the ten members of human TLRs by placental cells or cell lines has been reported, the expression of TLRs has been shown to be regulated in a temporal and time dependent manner during the human pregnancy period (11). For instance, TLR2 is expressed in the villous cytotrophoblasts of second-trimester placentas, but not in the syncytio-trophoblasts cells, whereas it is identified on syncytio-trophoblasts in third-trimester placentas (12). Besides, TLR6 has been reported to be expressed by third-trimester trophoblasts, while it is not expressed by first-trimester trophoblasts (13,14). Moreover, TLRs may play an important role in response to pregnancy-related stresses, such as placentation, labor, and delivery (15). TLRs stimulate and coordinate the process of human placentation and protect the fetus from infections (16,17,18). Indeed, activation of TLRs by their ligands in the first and third trimester placental cells induce secretion of cytokines and chemokines which play a significant role in the activation and migration of innate cells (19,20,21,22,23). In this process, chemokines and inflammatory cytokines, such as IL-1, IL-6, and TNF- $\alpha$ , are produced, enhancing the activity of neutrophils and monocytes (24). Ligation of TLRs on neutrophils, monocytes/macrophages, and NK cells activates the innate immune system and leads to the production of inflammatory cytokines as well as IFN- $\gamma$ . Uterine Natural Killer cells (uNK cells), dendritic cells, and macrophages play important roles in the maintenance of a normal pregnancy and creation of a uterine environment for normal embryo implantation and development (25). Excessive activation of the innate immune system induces severe inflammation and also Th1 type immunity. These immune conditions may cause pregnancy-related disorders, such as preeclampsia and abortion (24). In addition, many pregnancy-related disorders, such as PE, are thought to be associated with an increased innate inflammatory response and deviation of adaptive

immune responses toward Th1 type immunity (26). Interestingly, despite the important roles of TLRs in pregnancy and placental tissue development, a limited number of studies have been conducted on the expression and localization of TLRs in term normal placentas and pregnancy-related diseases. Thus, the present study aims to investigate the differences in the expressions of TLRs 2, 4, 5, 6 and a group of inflammatory-related cytokines, including IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$ , in different parts of placentas from PE and healthy pregnant women in their third trimester of pregnancy.

## MATERIALS AND METHODS

**Subjects.** The present study was done on 30 pregnant women who were in their third trimester of pregnancy (gestational age  $\geq$  37 weeks) after obtaining a written informed consent and gaining the approval of the local Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran for using the placentas and blood samples. In this study, 15 out of the 30 women were diagnosed with PE while the remaining 15 were healthy women without any history of hypertension, autoimmunity, or cancer diagnosis by the same gynecologist. Diagnosis of PE was based on the level of proteinuria and hypertension. Accordingly, a minimum of two blood pressure readings greater than 140/90 mmHg and proteinuria greater than 0.3 gr in a 24-hr urine specimen were considered as the mild form and above 160/110 mmHg blood pressure and more than 5 gr proteinuria were diagnosed as the severe form. Based on these criteria, five out of the 15 PE cases were diagnosed with severe and the remaining ten were diagnosed with the mild form. There were no significant differences between the healthy and preeclamptic women regarding age (mean age: 29.1 years in controls vs. 27.9 years in cases) and gestational age (mean: 38.1 weeks in controls vs. 37.6 weeks in cases). The women with the history of autoimmune or other immunological disorders (including infectious diseases, such as HIV) were excluded from the study.

**Sampling.** The placentas were collected after caesarean section. Immediately after the cesarean section, the cord bloods were collected and the maternal parts of the placentas were separated from the fetal parts. Approximately 500 mg of each tissue was collected, washed with cold normal saline, and stored in liquid nitrogen until extraction of total ribonucleic acid (RNA). Also, 5 ml peripheral blood was collected from all the participants before the section. Mononuclear cells were separated from cord and peripheral blood using the Ficoll hypaque method (Fresenius Kabi Norge AS, Norway) and stored in liquid nitrogen until extraction of total RNA.

**RNA Extraction and cDNA Synthesis.** Total RNA was extracted from all the fetal and maternal parts of the collected placentas using a total RNA extraction kit (Jena Bioscience GmbH, Jena, Germany) based on the manufacturer's instructions. Briefly, 500 mg of the tissue was powdered using liquid nitrogen before extraction. The same kit was used to extract total RNA from approximately  $3 \times 10^6$  separated cord and peripheral blood mononuclear cells. The concentration of the extracted RNA was determined using a Nano drop instrument. Moreover, the purity and quality of the extracted mRNA was checked using Agarose Gel Electrophoresis. All extracted RNAs were stored at  $-70^\circ\text{C}$  before synthesis of cDNA. cDNA was synthesized from 700 nanograms of total RNA using an easy<sup>TM</sup> cDNA synthesis kit (Pars Tous, Iran) based on the manufacturer's instructions. Briefly, the mixture of oligo-dT, RNA, and DEPC water was heated at  $65^\circ\text{C}$  for 5 minutes and was immediately transferred on ice. After adding RT premix

2X, cDNA was made and stored in aliquots at 70°C. The quality of cDNA was checked by PCR method.

**Quantitative Real-Time PCR (Q-PCR).** Quantitative real-time PCR (Q-PCR) was performed (Applied Biosystems 7500 Fast Real-Time PCR system) using the SYBR Green method to amplify TLR 2, 4, 5, 6 and IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$ . Briefly, 20 picomol of each primer (Table 1), 2  $\mu$ l of target cDNA (selected after checking and normalization of  $C_T$  values), and 10  $\mu$ l SYBR Green cocktail were used for amplification.

**Table 1. Primers Used for RT-PCR Analysis.**

Gene	Forward Primer	Reverse Primer	Product Size (bp)	Ref. no.
IFN- $\gamma$	CTAATTATTCGGTAACTGACTTGA	ACAGTTCAGCCATCACTTGGA	75	(30)
IL-1A	CCAACGGGAAGGTTCTGAAG	GGCGTCATTCAGGATGAATTC	191	(29)
IL-6	AAATTCGGTACATCCTCGAC	CCTCTTTGCTGCTTTCACAC	84	(28)
TNF- $\alpha$	GCCTGCTGCACTTTGGAGTG	TCGGGGTTCGAGAAGATGAT	108	(28)
TLR2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACTT	67	(27)
TLR4	CAGAGTTTCCTGCAATGGATCA	GCTTATCTGAAGGTGTTGCACAT	88	(27)
TLR5	TGCCTTGAAGCCTTCAGTTATG	CCAACCACCACCATGATGAG	77	(27)
TLR6	GAAGAAGAACAACCCCTTAGGATAGC	AGGCCAAAACAAAATGGAAGCTT	88	(27)
18s rRNA	CTCAACACGGGAAACCTCAC	AAATCGCTCCACCAACTAAGAA	114	(31)

IFN- $\gamma$ : Interferon gamma; IL-1A: Interleukin 1 alpha; IL-6: Interleukin 6; TNF- $\alpha$ : Tumor Necrosis Factor alpha; TLR: Toll-like receptor; 18s rRNA: 18s ribosomal RNA

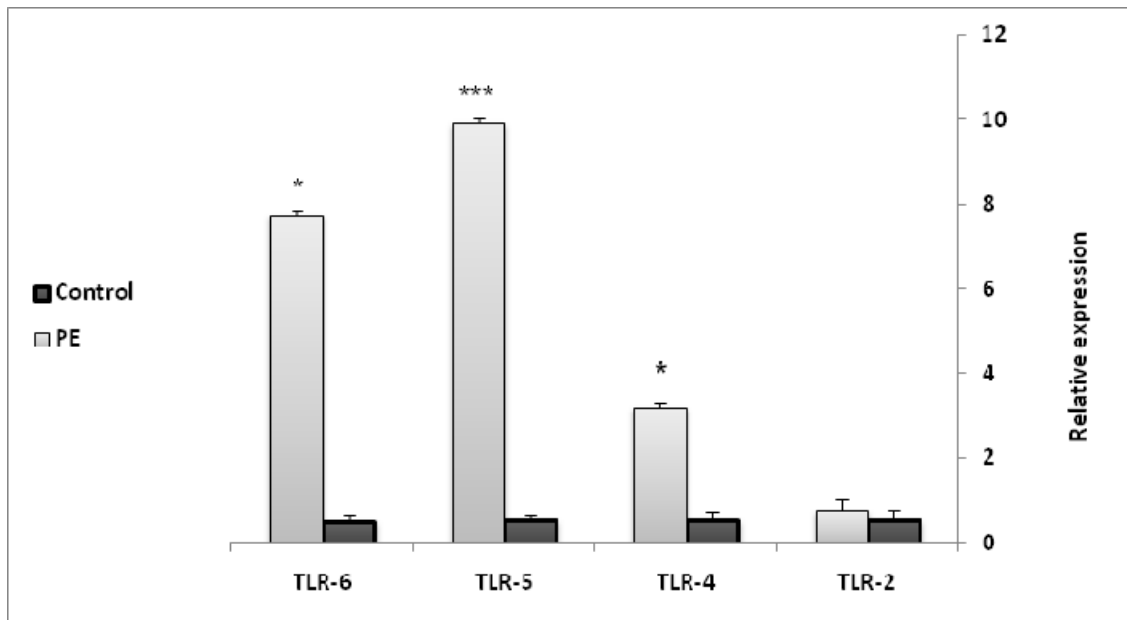
The final volume of each reaction was 20  $\mu$ l. Moreover, 18s rRNA was used as the housekeeping control gene. All the tests were done in duplicate and the mean  $\Delta C_T$  was used for calculation. The amplification condition consisted of an initial denaturation at 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s and annealing and amplification at 60°C for 35 s. Comparative  $C_T$  and fold differentiation ( $2^{-\Delta\Delta C_T}$ ) methods were used for quantification of target gene expression. Furthermore, calibrator sample and 18s rRNA (control housekeeping gene) were used to minimize the variation and normalize the Q-PCR, respectively.

**Statistical Analysis.** Mann Whitney Test was used for the quantification analysis of Q-PCR results by Statistical Package for the Social Sciences (SPSS) software, v. 16 (SPSS Inc, Chicago, IL, USA). P-values less than 0.05 were considered statistically significant.  $\Delta C_T$  values were used for comparison of the gene expression between the case and controls and  $2^{-\Delta\Delta C_T}$  was used for relative gene expression analysis.

## RESULTS

In general, the expressions of all studied TLRs and cytokines were detected in the placental samples of the healthy and preeclamptic women. However, not all the blood samples showed expressions of all the studied cytokines.

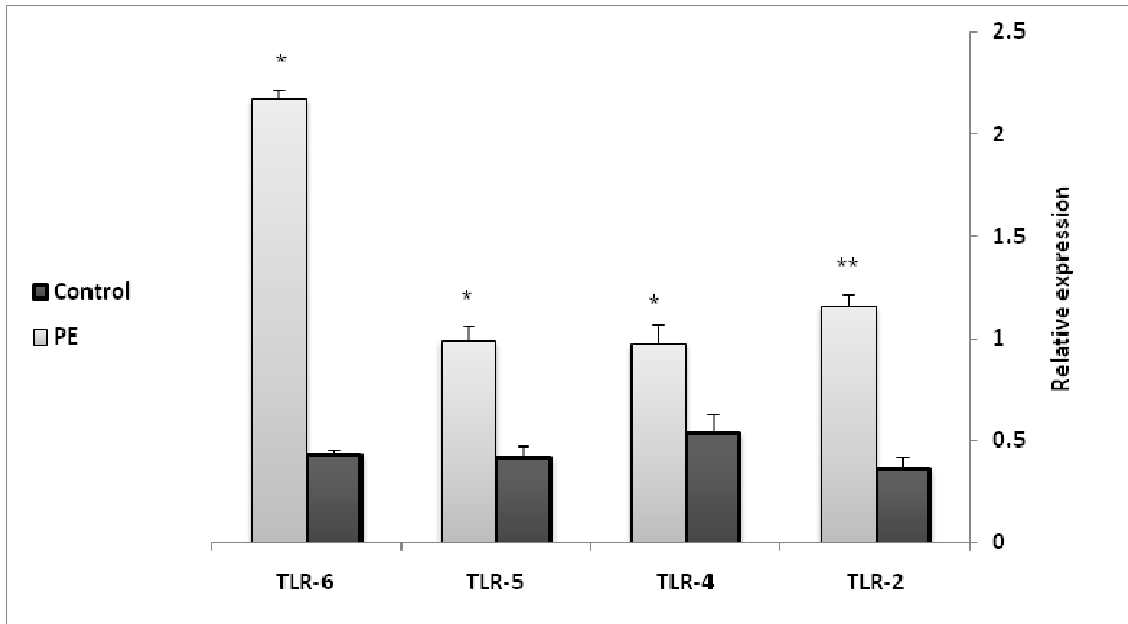
**Difference in the Expression of TLRs between Healthy and Preeclamptic Placentas.** The expressions of TLRs 2, 4, 5, and 6 showed up regulation in the preeclamptic compared to the healthy placentas. Comparison of the expression pattern of the studied TLRs between the maternal part of the preeclamptic and healthy placentas indicated that except for TLR2, all the other studied TLRs were significantly over expressed in preeclamptic placentas ( $p < 0.001$  for TLRs 4,6 and  $p < 0.003$  for TLR 5, Figure 1).



**Figure 1.** Histograms depicting a comparison between the relative expression of transcripts for TLR-2, TLR-4, TLR-5 and TLR-6 in the maternal parts of the placenta from preeclamptic and healthy women (versus 18s rRNA). \* $p < 0.001$ , \*\* $p < 0.002$ , \*\*\* $p < 0.003$ .

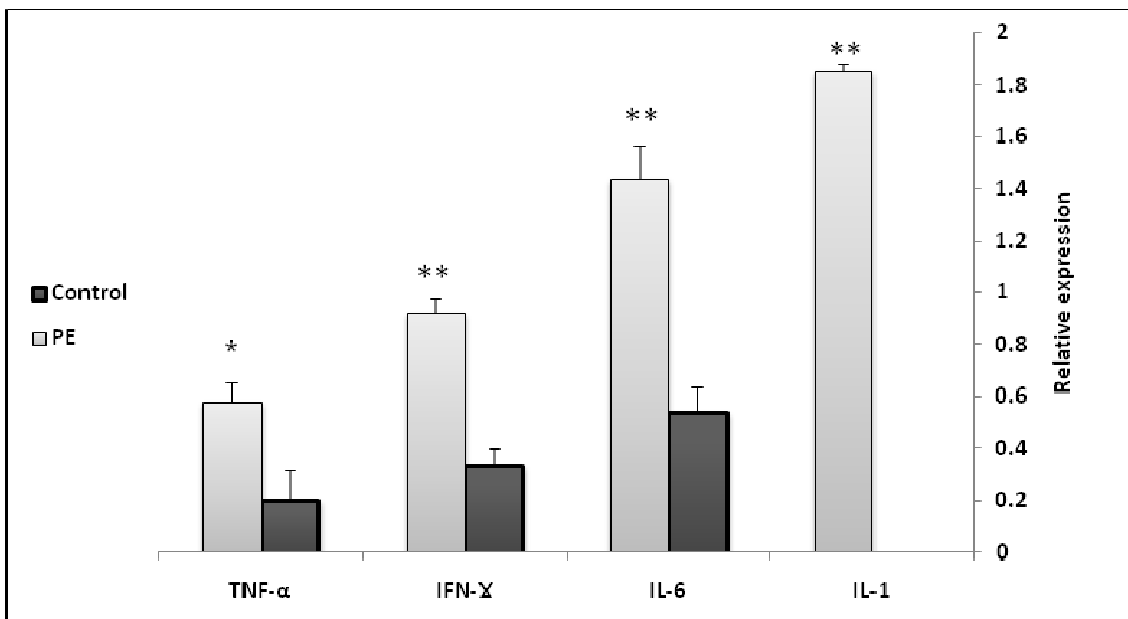
Regarding the fetal part of the placenta, all studied TLRs showed over expression in the preeclamptic placentas compared to the healthy ones ( $p < 0.001$  for TLRs 4, 5, 6 and  $p < 0.002$  for TLR 2, Figure 2).

**Difference in the Expression of the Inflammatory Cytokines between Healthy and Preeclamptic Placentas.** Comparison of the expressions of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  at RNA level in preeclamptic and healthy placentas indicated that the expressions of inflammatory cytokines were up regulated in both the maternal ( $p < 0.006$  for TNF- $\alpha$  and  $p < 0.001$  for IL-1, IL-6 and IFN- $\gamma$ , Figure 3) and fetal ( $p < 0.001$  for all the studied cytokines, Figure 4) parts of the preeclamptic placentas compared with healthy ones.

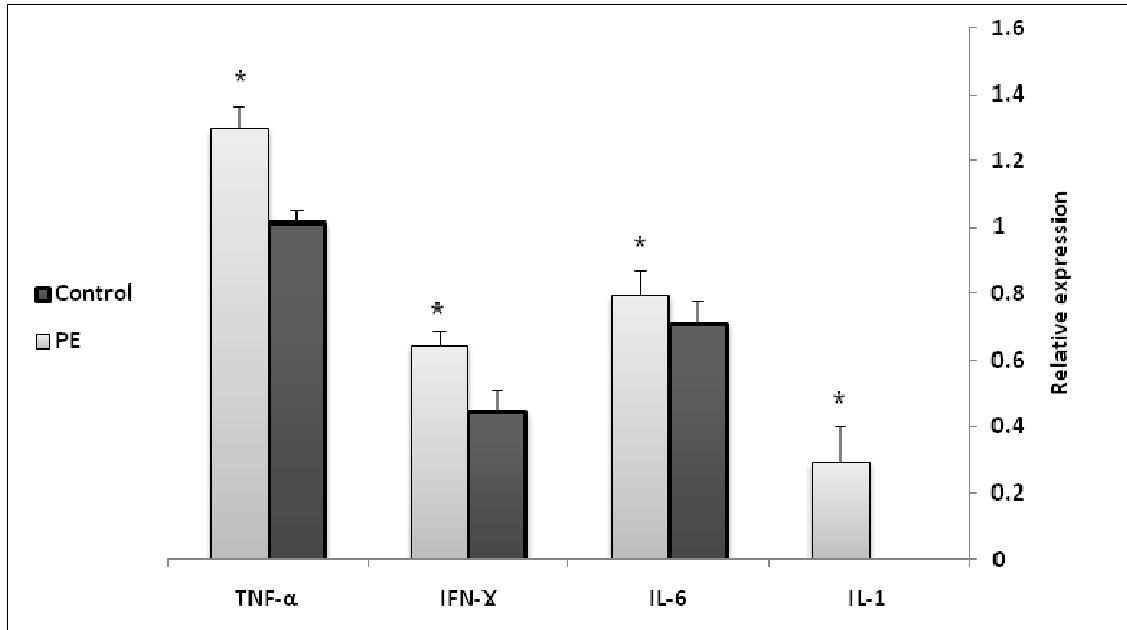


**Figure 2.** Histograms depicting a comparison between the relative expression of transcripts for TLR-2, TLR-4, TLR-5 and TLR-6 in the fetal parts of the placenta from preeclamptic and healthy women (versus 18s rRNA). \*p < 0.001; \*\*p < 0.002; \*\*\*p < 0.003.

However, there were some differences between the maternal and fetal parts of the PE placentas regarding the expression of the studied cytokines.

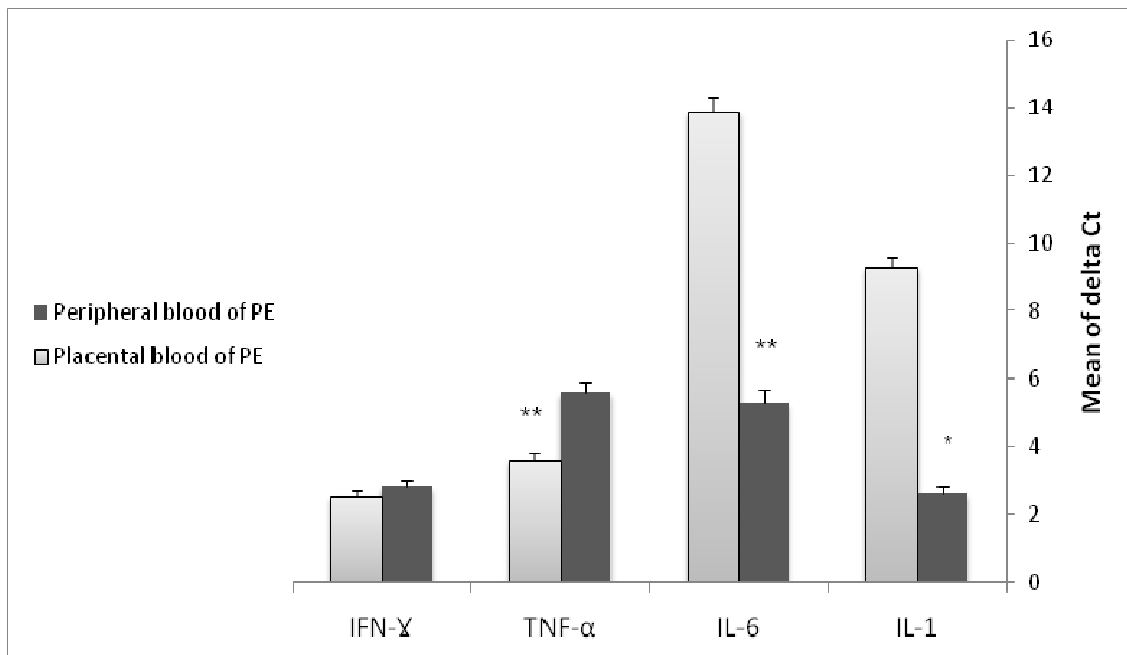


**Figure 3.** Histograms depicting a comparison between the relative expression of transcripts for IL-1, IL-6, TNF-α and IFN-γ in the maternal parts of the placenta from preeclamptic and healthy women (versus 18s rRNA). \*p < 0.006; \*\*p < 0.001.



**Figure 4.** Histograms depicting a comparison between the relative expression of transcripts for IL-1, IL-6, TNF- $\alpha$  and IFN- $\gamma$  in the fetal parts of the placenta from preeclamptic and healthy women (versus 18s rRNA). \*  $p < 0.001$ .

IL-1 and IL-6 were both significantly over-expressed in the fetal parts of the PE placentas compared with the maternal parts ( $p < 0.001$  for IL-6 and  $p < 0.02$  for IL-1, Figure 5) while TNF- $\alpha$  showed over-expression in the maternal parts ( $p < 0.001$ , Figure 5).



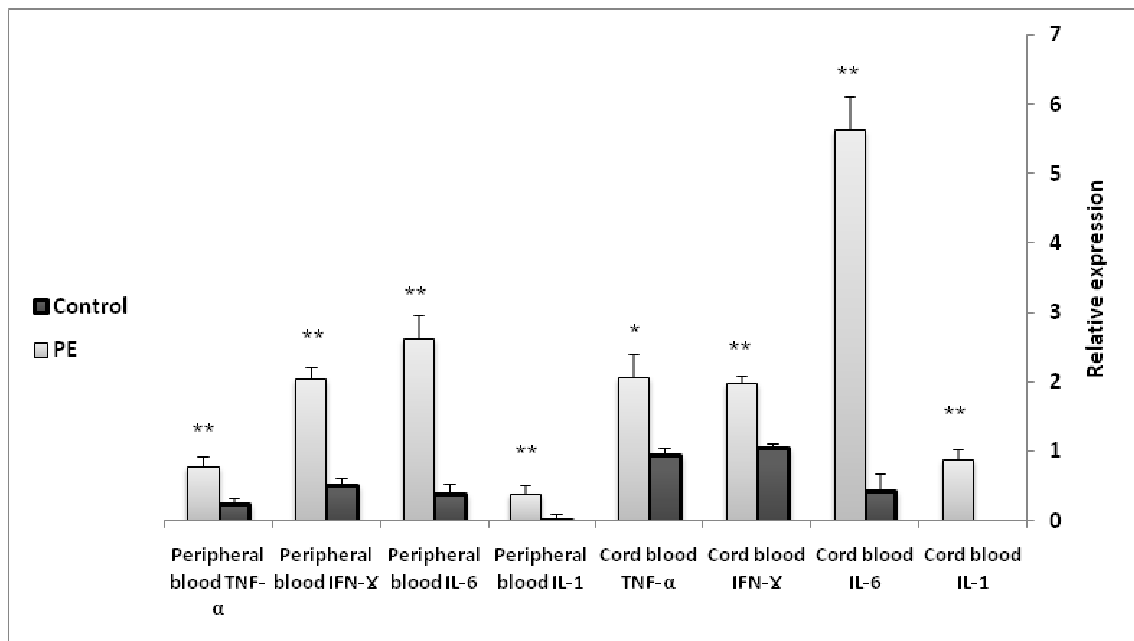
**Figure 5.** Histograms depicting a comparison between the relative expression of the studied cytokines in peripheral and placental blood samples from PE patients.

\*  $P < 0.02$ , \*\*  $p < 0.001$

Moreover, most of the fetal part samples from healthy placentas express the detectable level of IL-6 and TNF- $\alpha$  while most of the maternal parts did not express IL-6 or TNF- $\alpha$ .

**Difference in the Expression of Inflammatory Cytokines between Healthy and Preeclamptic Blood Samples.** As observed in the placental samples, preeclamptic women produced higher levels of inflammatory cytokines in comparison to healthy women. Moreover, the expression patterns of the studied cytokines were almost the same in peripheral and cord blood samples. Regarding IL-1, although a low level of this cytokine was detected in all cord and peripheral samples from the preeclamptic women, the expression of IL-1 was detected in only one healthy peripheral blood sample. Nevertheless, expression of IL-1 was not detected in any of the cord blood samples ( $p < 0.001$  for both cord and peripheral blood samples, Figure 6).

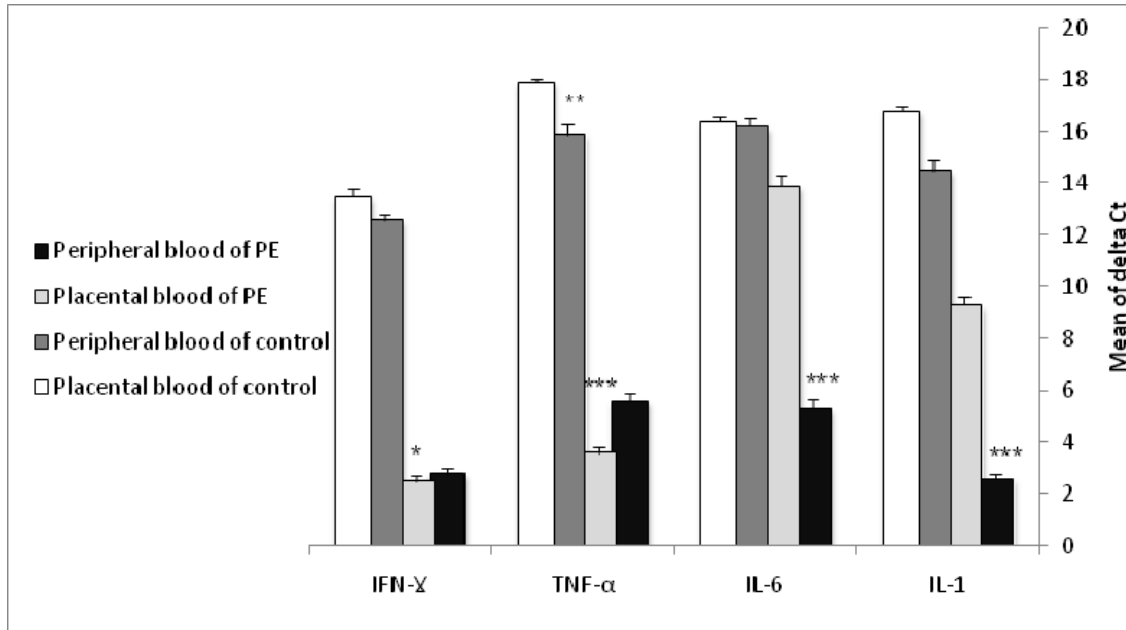
Furthermore, low expression of IL-6 was detected in 11 peripheral and 7 cord blood samples from the healthy women, while all the patients expressed IL-6 at RNA level ( $p < 0.001$  for both cord and peripheral blood sample, Figure 6). Additionally, 12 out of the 15 healthy women produced a detectable level of TNF- $\alpha$ , while this cytokine was significantly up regulated and detected in all patient samples ( $p < 0.004$  for peripheral and  $p < 0.001$  for cord blood samples, Figure 6).



**Figure 6.** Histograms depicting a comparison between the relative expression of transcripts for IL-1, IL-6, TNF- $\alpha$  and IFN- $\gamma$  in the cord and peripheral blood from preeclamptic and healthy women (versus 18s rRNA). \*\* $p < 0.001$ ; \* $p < 0.004$ .

The expression level of IFN- $\gamma$  was also significantly up regulated in the PE cases compared to healthy women ( $p < 0.001$  for both cord and peripheral blood samples, Figure 6).





**Figure 7.** Histograms depicting a comparison between relative expression of the studied cytokines in peripheral and placental blood samples from preeclamptic and healthy women \* $p < 0.04$ , \*\* $p < 0.004$ , \*\*\* $p < 0.001$ .

When the expression of the studied cytokines within peripheral and cord blood were compared in PE patients, IL-1 and IL-6 showed over expression in the peripheral blood ( $p < 0.001$ , Figure 7) while TNF- $\alpha$  and IFN- $\gamma$  showed over expression in the cord blood samples ( $p < 0.001$  and  $p < 0.04$  respectively, Figure 7).

Comparison of the cytokine expression between the peripheral and placental blood samples from healthy women indicated that, except for TNF- $\alpha$ , there were no significant differences between the two samples regarding expression of the studied cytokines. TNF- $\alpha$  in healthy women showed significant over-expression in the peripheral blood samples ( $p < 0.004$ , Figure 7).

## DISCUSSION

In this study, for the first time, we simultaneously compared the expressions of a group of TLRs and inflammatory cytokines in two different parts of human full term placentas. We also investigated and compared the expression profiles of inflammatory cytokines in cord and peripheral blood samples from preeclamptic and healthy term women. Several studies have indicated that innate immune responses play a critical role in the pathogenesis of PE (11,24). Indeed, PE is a state of excessive innate inflammatory responses, but what truly triggers such an excessive inflammation is not clear, yet. TLR stimulation is one of the most important triggering signal for production of inflammatory cytokines. As indicated in the present and previous studies, although normal full term placentas express TLRs, it seems that TLRs are expressed more by preeclamptic placentas (32). In spite of the fact that infectious agents are the best known ligands and stimulators for TLRs expression, in the absence of infection, other ligands

such as apoptotic cells or stress proteins may trigger over-expression of TLRs. Interestingly, it has been reported that placentas from the women diagnosed with PE show an increased trophoblast apoptosis rate compared to the normal term placentas (33). Whatever are the trigger factors or ligands, TLRs ligation leads to the activation of production of pro-inflammatory cytokines via the NF- $\kappa$ B pathway (34-36) and chronic inflammation is considered to be a major contributor to the development of PE (37). In line with the previous studies, the results of the present study indicate that the preeclamptic placentas generally over-express the TLRs. However, some differences were found between the maternal and fetal parts of the placentas regarding TLRs expression. Although TLRs 4, 5, and 6 were all significantly over-expressed in the maternal part of the preeclamptic placentas, no significant difference was observed between preeclamptic and healthy placentas regarding the expression of TLR2. This finding is consistent with that of some previous reports regarding the expression of TLR2 in preeclamptic placentas (33). Concerning the fetal part of the placentas, all the studied TLRs indicated over-expression in preeclamptic ones compared with healthy placentas.

The relationship between over-expression of TLRs and production of inflammatory cytokines has been well documented (16). Indeed, infectious and non-infectious ligands promote the expression of the inflammatory cytokines via activation of TLRs. Up to now, most of the studies have investigated the expression of the inflammatory cytokines in the total placental tissue. In the present study however, we tried to investigate the difference in expression of these cytokines in both maternal and fetal parts of the placenta. Human pregnancy is a systemic inflammatory status characterized with the expression of inflammatory cytokines, such as IL-1, IL-6, and TNF- $\alpha$  (38). Both maternal immune cells and trophoblast cells produce inflammatory cytokines during pregnancy (16,39). Nonetheless, it seems that the inflammatory responses are controlled in a way that does not harm the mother or the fetus. In preeclampsia, on the contrary, the inflammatory cytokines are over-expressed, resulting in the incidence of the clinical signs and symptoms of PE. Although our results were in line with the findings regarding the upregulation of the inflammatory cytokines in PE cases, some differences were observed between the fetal and maternal parts of the placentas. The relationship between over-expression of TLR4 and TLR6 and up regulation of IL-1 has been reported in pregnancy-related diseases (27,40,41). In agreement with these findings, our results also indicated a correlation between TLR4 and TLR6 expression and IL-1 level in both the maternal and fetal parts of the placentas. Regarding IFN- $\gamma$ , in line with the previous reports, the level of this cytokine showed an increased expression in both fetal and maternal parts of the preeclamptic placentas. It has been proposed that infection with bacterial and viral agents during pregnancy might be involved in the etiology of PE. TLR-4 signaling had an important role in the response to intracellular bacterial infection or endogenously generated inflammatory ligands via induction of IFN- $\gamma$  (42). Our results regarding the over-expression of TLR4 along with IFN- $\gamma$  in both fetal and maternal parts of the placenta are also in agreement with this hypothesis. Although the over-expression of IL-6 and TNF- $\alpha$  by preeclamptic placentas were reported before (43), our results indicated that the major source of IL-6 and TNF- $\alpha$  in a healthy third trimester placenta is the fetal part, while in the PE patients, fetal parts expressed more IL-6 and the maternal parts expressed more TNF- $\alpha$ . So it seems that different parts of the placentas play different role in induction of the inflammatory status within the placenta and this role may differ in healthy and PE placentas. In addition to its several

roles in placental development, TNF- $\alpha$  plays an important role in the control of delivery (44). The findings of the current study show that the inflammatory responses due to the up-regulation of TNF- $\alpha$  and IL-6 in a normal pregnancy are mainly supported by fetal TNF- $\alpha$  and IL-6. In preeclamptic patients, the maternal parts of the placentas actively participate in the induction of the exaggerated placental inflammatory status by production high levels of TNF- $\alpha$ . Therefore, it seems that over expressed TNF- $\alpha$  and IL-6 from both maternal and fetal parts work together to generate the increased inflammation in PE placentas. In this study, assessment of IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  within both peripheral and cord blood samples supported the results obtained from the investigation of the placental tissue samples, and indicated that PE is a systemic inflammatory disorder. However, comparison of the cytokines levels between peripheral and cord bloods in PE patients, indicated that TNF- $\alpha$  and IFN- $\gamma$  were more concentrated in the cord, whereas, IL-1 and IL-6 were more over-expressed in the peripheral blood samples. Overall, our results of the evaluation of the cytokines in placental and cord bloods and placental tissue samples indicated that the assessment of the inflammatory cytokines within different parts of the placental tissue or cord and peripheral blood samples would provide a better insight as to what goes on during normal pregnancy and pregnancy-related disorders, such as PE. In conclusion, the findings of the present study indicate that the expression of TLRs and inflammatory cytokines in the pregnancy period is a dynamic process. Moreover, it seems that the expression of the studied TLRs and cytokines are controlled in normal pregnancy, but are up regulated in the case of PE. Finally, differences were observed between the maternal and fetal parts of the placentas as well as peripheral and cord blood samples in contribution to the inflammatory status of the placenta. Yet, the role of different parts of the placenta and the trigger factors for such over-expressions or protective and adverse effects of the innate immune system activation during the pregnancy period remain to be further investigated in future studies.

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