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Cytokine secretion by decidual lymphocytes in transient hypertension of pregnancy and pre-eclampsia

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Background: **Transient hypertension (TH) and pre-eclampsia (PE) are believed to have different pathophysiology. However, 15–25% of pregnant women initially diagnosed as having TH develop PE. To clarify the immuno-pathogenetical connections between the two syndromes, we studied the pattern of T helper cell (Th)1/Th2 cytokine balance disturbances existing inside maternal decidua in normal pregnancy (NP) and pregnancies complicated with TH and PE.**

Methods: **Third-trimester decidual tissue was obtained by curettage of uterine cavity during elective caesarean sections in NP (n = 11), TH (n = 17) and PE (n = 21) patients. Cell suspensions were prepared by an electromechanical dispersal method and centrifugated using a standard gradient sedimentation technique. Isolated lymphocytes were placed in medium (RPMI 1640, 10% fetal calf serum, L-glutamine, penicillin, streptomycin) and cultured for 72 h with or without mitogen phytohaemaglutinine (PHA). The enzyme-linked immunosorbent assay method was used for estimation of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12 and interferon-γ (IFNγ) in culture supernatant.**

Statistical analysis: **The Kruskal–Wallis and the Mann–Whitney U tests were used ($p < 0.05$).**

Results: **Both spontaneous and PHA-stimulated secretion of Th2-type cytokines IL-6 and IL-10 was decreased in PE patients compared with TH and NP patients. The concentration of Th1-type cytokine IFNγ was increased in patients suffering both from TH and PE.**

Conclusion: **On the base of decidual cytokine secretion, both PE and TH are syndromes of local Th1/Th2 cytokine balance disturbances as compared with NP, and TH seems to be an intermediate step to PE.**

Key words: Lymphocyte, Cytokine, Decidua, Pre-eclampsia, Transient hypertension

Introduction

Although the clinical presentations of pre-eclampsia–eclampsia and transient hypertension of pregnancy at least partially overlap, opinion exists that they are two distinct conditions[1] with different pathophysiology[2–5] and outcome.[6] However, epidemiological analysis indicates that approximately 15–25% of pregnant women initially diagnosed as having transient hypertension develop fully symptomatic pre-eclampsia later in pregnancy.[7] This means that, in some patients, transient hypertension could probably represent an intermediate step in progression to pre-eclampsia.

The involvement of abnormal activation of the innate and acquired immune system in the pathogenesis of pre-eclampsia is well documented.[8,9] The elevated serum level of interleukin (IL)-12,[10] fetal monocyte activation[9] and deficiency of placental IL-10 production[11] are responsible for enhanced T helper (Th)1-type cell maturation and hampered immunological tolerance of pre-eclamptic women to the foreign antigens of the fetus. The existence of a superantigen-like effect in a subset of patients[12] supports the idea of the immunopathogenetical background of pre-eclampsia.

A widely accepted hypothesis originally presented by Wegman *et al.*[13] underlines the crucial role of Th2-activity cytokines in promoting successful pregnancy. Since that time, existence of the 'Th2-phenomenon' during normal human pregnancy has been confirmed by many authors.[14–18] Pre-eclampsia is accompanied with elevated serum levels of Th1-type cytokines IL-12[10] and tumor necrosis factor-α(TNFα),[19] as well as Th2-type cytokine IL-6.[19] Upregulation of Th1-activity responses was also shown '*in vitro*' by increased interferon-γ (IFNγ),[14] IL-2 and TNFα[14,20] production by peripheral blood lymphocytes. IL-2 and IL-12 stimulate Th1-like cell maturation[21,22] and TNFα may

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contribute for endothelial activation and failure observed in pre-eclampsia.[19] The predominance of Th1-type activity was accompanied by the effect of decreased Th2-type suppressory cytokine IL-10 secretion by peripheral blood lymphocytes '*in vitro*'[20] as well as in placental samples.[11] The investigations performed during pregnancy complicated with pre-eclampsia indicated that disturbances of cytokine Th1/Th2 balance are associated with elevated endothelin-1 levels followed by the presence of hypertension.[23]

None of the aforementioned studies refered to the autocrine pattern of Th1/Th2 cytokine balance inside maternal decidua in pre-eclampsia and transient hypertension, and their potential role in understanding the local regulatory mechanisms seen in those two syndromes. To clarify this subject, we have focused our attention on comparison between spontaneous and mitogen-stimulated production of IL-2, IL-4, IL-6, IL-10, IL-12 and IFNγ in cultured lymhocytes isolated from third trimester decidua of healthy pregnant women, pre-eclamptics and those pregnant with transient hypertension. The obtained results have suggested that disturbances in autocrine regulation may be decisive for disease outcome.

Materials and methods

Patients

The study group was chosen from pregnant women who were hospitalized between September 1998 and January 2001 in three departments: the Department of Materno-Fetal Medicine, the Department of Perinatology and the Department of the Obstetrics of Polish Mother's Health Center Research Institute, Lodz, Poland.

Inclusion criteria were normal pregnancy, and pregnancy complicated with transient hypertension or pre-eclampsia. Pre-eclampsia was characterized by an increase in systolic pressure of 30 mmHg or of diastolic pressure of 15 mmHg compared with blood pressure measurements obtained before 20 gestational weeks (or, if these pressure levels were not known, a blood pressure of 140/90 mmHg or greater obtained in two consecutive measurements 6 h apart after 20 gestational weeks), with concurrent proteinuria greater than 0.3 g per 24 h or greater than 30 mg/dl in a specimen. Transient hypertension was characterized by the same blood pressure measurements but without proteinuria or with proteinuria less than 0.3 g per 24 h.[1]

Exclusion criteria were diabetes mellitus, gestational diabetes mellitus, renal diseases, chronic hypertension that predated pregnancy, infectious diseases recognized in the course of pregnancy, presence of premature or full-term uterine contractions, premature rupture of membranes or clinical chorioamnionitis. The pregnant women were not treated with corticosteroids before inclusion for the study. Establishment of such a wide range of exclusion criteria allowed us to eliminate possible clinical situations that could have a strong impact either on the composition of decidual lymphocytes or on the pattern of cytokine secretion.

Finally, 49 pregnant women were chosen for the study and, in all cases, the selection was made before decidual material was collected. All of them had been qualified for elective caesarean sections. In this group, 11 pregnant women had normal pregnancy (controls), 17 presented with transient hypertension of pregnancy and 21 with pre-eclampsia. Some of the data characterizing each group are presented in Table 1. In the group of controls, the indications for elective caesarean sections were retinal degeneration changes, serious cardiac defects, pelvic deformations, cerebral vessels malformations, multifetal pregnancy and breech position in women with high-risk pregnancy. In the group of transiently hypertensive patients the most common indication

Table 1. Data from routine interview, clinical investigation, blood pressure measurements and laboratory testing in healthy pregnant women, and pregnant patients with transient hypertension and pre-eclampsia

Parameter	Normal pregnancy (n = 11)	Transient hypertension (n = 17)	Pre-eclampsia (n = 21)
Patient age (years)	29.5 ± 5.7	28.6 ± 5.3	28.7 ± 6.1
Gestational age (weeks)	36.5 ± 4.9	35.1 ± 3.5	33.3 ± 2.7
Primigravidae (n)	8	13	16
Mean systolic pressure (mmHg)	127.0 ± 7.8	171.9 ± 15.8	178.5 ± 21.9
Mean diastolic pressure (mmHg)	78.7 ± 7.7	107.1 ± 10.8	111.6 ± 12.3
Proteinuria (mg/dl)	–	28.3 ± 2.9	509.9 ± 851.7
Proteinuria/24 h (g/24 h)	–	–	4.9 ± 6.1
Serum uric acid (mg/dl)	3.8 ± 1.3	5.7 ± 2.0	7.0 ± 1.9
Platelet count (G/l)	235.7 ± 43.8	208.8 ± 40.9	204.7 ± 55.6
Serum aspargin aminotransferase (IU/l)	27.9 ± 4.1	40.7 ± 19.8	54.5 ± 30.8
Serum alanin aminotransferase (IU/l)	17.4 ± 15.2	27.6 ± 13.2	62.4 ± 55.2

Data presented as mean ± standard deviation.

was severe hypertension complicated with cardiotocographic signs of fetal distress, while in pre-eclamptic patients the indications were symptoms of imminent eclampsia and/or cardio-tocographic signs of threaten fetal asphyxia. All patients gave written and informed consent for participation in clinical research and the agreement of the Polish Mother's Health Center Research Institute Ethical Commitee for performing the study was obtained.

Cell isolation

Third-trimester maternal decidual tissue was obtained by curettage of uterine cavity during caesarean sections. The blood clots and fragments of the fetal membranes were removed macroscopically using sterile pincers. Then the samples were placed into bottles containing sterile phosphate-buffered saline (PBS) (WSS, Lublin, Poland). Some of the collected samples after initial washing in PBS were randomly submitted to histopathological examination, which revealed that trophoblastic villi were present in less than 5% of the specimen volume, giving the certainty that samples contained almost pure decidua. Inside laminar flow cabinet decidual slices were rinsed several times in PBS to remove residual blood and than mechanically disaggregated to fragments of approximately 3–10 mm^3 volume. Subsequent decidual cell suspensions were prepared by an electromechanical dispersal method using Medimachine (Dako, Copenhagen, Denmark). Tissue fragments with 1.5 ml PBS were placed in the Medicon disaggregator chamber (Dako) with 50 μm of separator mesh. The optimal time of disaggregation was 20 sec. Cell suspensions were filtered using disposable sterile Filcon (Dako) with a 50–70 μm pore size range and washed twice to eliminate cell debris. To isolate lymphocytes, cell suspensions were centrifugated using the standard gradient sedimentation technique on Gradisol G (Polfa, Kutno, Poland). Isolated lymphocytes were washed and suspended in PBS in a final density of 1.0×10^5 cells/ml. The viability of the cells in suspension was about 98%, as tested with the trypan-blue exclusion method.

Lymphocyte subset analysis

Monoclonal antibodies labeled with fluorescein isothiocyanate (FITC) or phycoerytrin (PE) were used in the study. One-color or two-color immunofluorescence staining was performed with the use of the following antibodies (Becton-Dickinson, San Jose, CA, USA): anti-CD3 (against mature T lymphocytes), anti-CD19 (against B lymphocytes), anti-CD4/CD8 (against helper/inducer and suppressor/cytotoxic lymphocytes), and anti-CD56/CD16 (against natural killer (NK) cells). Flow cytometry was performed on FACSCalibur (Beckton-Dickinson) with a 488 nm argon laser. Optimal scatter gates were set using LeucoGATE (anti-CD45 FITC and anti-CD14 PE; Becton-Dickinson) so that analysis gate for cells included more than 96% of lymphocytes and less than 3% of monocytes/granulocytes. For background control, immunoglobulin (Ig)G1 FITC and IgG2a PE (Becton-Dickinson) were used. No less than 1×10^4 cells were measured in each analysis. The results are presented as the percentage of positive cells in the tested sample.

Cytokine production

Isolated decidual lymphocytes (1.0×10^5 cells/ml) were placed in culture medium: RPMI 1640 (Flow) enriched with 10% fetal calf serum (Sigma, St Louis, MO, USA), 2 mM L-glutamine (Serva, Heidelberg, Germany), and antibiotics (100 U/ml of penicillin, 10 μg/ml of streptomycin). To stimulate lymphocyte proliferation, phytohaemaglutinine (PHA) (Sigma) in a concentration of 5 μg/ml of culture medium was used. Cultures were conducted in flat-bottomed microplates (Nunc, Kamstrupvij, Denmark) using 10^5 cells/100 μl of medium, for 72 h in humidified air with 5% CO_2 (Assab, Stockholm, Sweden), and each experiment carried out in triplicate. Control triplicates had no mitogen added. At the end of the culture, microplates were centrifugated at $2000 \times g$ for 10 min. Then the culture supernatant was collected and frozen at –80°C. A standard immunoenzymatic enzyme-linked immunosorbent assay method was used for estimation of IL-2, IL-6, IL-10, IL-12, IFNγ (ENDOGEN, Minneapolis, MN, USA) and IL-4 (Hybridomus; Cytotech, Copenhagen, Denmark) concentrations. The results are presented as picograms per milliliter. The minimal detection limits for cytokines were as follows: < 6 pg/ml for IL-2, 1.1 pg/ml for IL-4, < 1 pg/ml for IL-6, < 3 pg/ml for IL-10, < 5 pg/ml for IL-12, and < 2 pg/ml for IFNγ. Intra-assay and inter-assay coefficients of variation for all studied cytokines were < 10%.

Statistical analysis

Results are reported as the group median with cut-off points of 25% and 75% of results. Analysis of differences between three groups was initially carried out using the Kruskal–Wallis test. Than the Mann–Whitney U test ($p < 0.05$) was performed as appropriate to test for the statistical significance of differences between each pair of groups. We used licensed Statistica 5.0 for Windows.

Results

The examples of the main maternal lymphocyte subsets obtained during isolation from third-trimester decidual tissue are presented in Table 2. Pre-eclamptic patients, compared with controls and those pregnant and with transient hypertension, were characterized with significantly increased percentage of classical

Table 2. The percentage of main decidual lymphocyte subsets in the group of healthy pregnant women, and pregnant patients with transient hypertension and pre-eclampsia

Decidual lymphocyte subsets (%)	(1) Normal pregnancy (n = 11)	(2) Transient hypertension (n = 17)	(3) Pre-eclampsia (n = 21)	p
$CD3^+$	58.0 (55.0/62.0)	56.0 (49.0/62.0)	32.0 (26.0/50.0)	(1)–(2), NS (1)–(3), < 0.0004 (2)–(3), < 0.0006
$CD19^+$	3.0 (2.0/5.0)	3.0 (2.0/4.0)	1.0 (1.0/2.0)	(1)–(2), NS (1)–(3), < 0.02 (2)–(3), < 0.005
$CD4^+$	25.0 (22.0/28.0)	26.0 (24.0/31.0)	19.0 (13.0/26.0)	(1)–(2), NS (1)–(3), NS (2)–(3), NS
$CD8^+$	26.0 (24.0/30.0)	23.0 (20.0/28.0)	26.0 (24.0/36.0)	(1)–(2), < 0.04 (1)–(3), NS (2)–(3), < 0.05
NK cells	35.0 (32.0/38.0)	24.0 (20.0/33.0)	57.0 (45.0/62.0)	(1)–(2), < 0.02 (1)–(3), < 0.002 (2)–(3), < 0.00003

Data presented as median (25%/75%). $CD3^+$, Lymphocyte T; $CD19^+$, lymphocyte B; $CD4^+$, helper/inducer lymphocytes; $CD8^+$, suppressor/cytotoxic lymphocytes; NK cells, classical natural killer $CD3^-/CD56^+CD16^+$ cells; NS, difference statistically unsignificant.

NK $CD3^-/CD56^+16^+$ cells and decreased percentage of mature $CD3^+$ T and $CD19^+$ B lymphocytes.

The concentrations of cytokines secreted spontaneously to supernatant of cultured decidual lymphocytes are compared in Table 3 and presented graphically in Figs 1, 3, 5, and 7. Analysis indicated significantly decreased levels of IL-6 and IL-10 in pre-eclamptic patients as well as increased level of IFNγ in patients suffering from both transient hypertension and pre-eclampsia.

The concentrations of cytokines secreted on mitogen stimulation of cultured decidual lymphocytes are presented in Table 4 and presented graphically in Figs 2, 4, 6, and 8. Lymphocytes of pre-eclamptic women secreted significantly decreased amounts of IL-6, IL-10 and IL-12 but extremely increased amounts of IFNγ.

Table 3. Spontaneous cytokine secretion (pg/ml) from cultured decidual lymphocytes of normal pregnant women, and pregnant patients with transient hypertension and pre-eclampsia

Cytokine	(1) Normal pregnancy (n = 11)	(2) Transient hypertension (n = 17)	(3) Pre-eclampsia (n = 21)	p
IL-2	0.0 (0.0/0.0)	0.0 (0.0/5.9)	0.0 (0.0/0.0)	(1)–(2), NS (1)–(3), NS (2)–(3), NS
IL-4	0.0 (0.0/0.0)	0.0 (0.0/0.0)	0.0 (0.0/0.0)	(1)–(2), NS (1)–(3), NS (2)–(3), NS
IL-6	1661.5 (1115.0/1957.0)	1454.2 (1165.0/4090.0)	720.0 (111.2/1147.0)	(1)–(2), NS (1)–(3), < 0.002 (2)–(3), < 0.0008
IL-10	115.0 (53.3/267.5)	128.5 (9.4/394.0)	41.0 (12.5/89.0)	(1)–(2), NS (1)–(3), < 0.05 (2)–(3), NS
IL-12	16.0 (0.0/17.8)	12.0 (3.3/16.9)	12.5 (0.0/23.7)	(1)–(2), NS (1)–(3), NS (2)–(3), NS
IFNγ	0.0 (0.0/0.5)	7.6 (0.0/35.4)	3.9 (0.0/21.0)	(1)–(2), < 0.03 (1)–(3), 0.05 (2)–(3), NS

Data presented as median (25%/75%). NS, Difference statistically unsignificant.

Table 4. PHA-stimulated cytokine secretion (pg/ml) from cultured decidual lymphocytes of normal pregnant women, and pregnant patients with transient hypertension and pre-eclampsia

Cytokine	(1) Normal pregnancy (n = 11)	(2) Transient hypertension (n = 17)	(3) Pre-eclampsia (n = 21)	P
IL-2	565.4 (285.2/802.9)	311.0 (8.6/700.6)	624.0 (316.9/960.8)	(1)–(2), NS (1)–(3), NS (2)–(3), NS
IL-4	17.8 (14.8/39.1)	51.5 (21.3/78.6)	30.0 (17.2/48.6)	(1)–(2), 0.06 (1)–(3), NS (2)–(3), NS
IL-6	7211.0 (6186.0/7990.0)	7559.5 (5930.0/9268.7)	3295.2 (582.0/6416.4)	(1)–(2), NS (1)–(3), < 0.02 (2)–(3), < 0.002
IL-10	1046.2 (645.6/1548.2)	1015.0 (330.0/1865.0)	282.0 (155.2/680.0)	(1)–(2), NS (1)–(3), < 0.002 (2)–(3), < 0.008
IL-12	436.6 (151.0/491.2)	209.8 (92.4/417.0)	212.7 (79.6/300.4)	(1)–(2), NS (1)–(3), < 0.05 (2)–(3), NS
IFNγ	2950.3 (1018.9/13249.2)	24850.0 (3105.8/45850.0)	44201.0 (41980.0/76450.0)	(1)–(2), 0.06 (1)–(3), < 0.0002 (2)–(3), < 0.02

Data presented as median (25%/75%). NS, Difference statistically unsignificant.

Discussion

Our study, in concordance with previous investigations performed on cultured peripheral blood mononuclear cells (PBMCs),[14,20] revealed that also lymphocytes isolated from decidua of pre-eclamptic patients are capable of producing, spontaneously and after PHA stimulation, large amounts of IFNγ. IFNγ belongs to cytokines of strong Th1-like activity and is mainly produced by T lymphocytes and activated decidual NK cells.[22] Because PHA is believed to exert its mitogenic effects mainly on T lymphocytes, it is possible that increased levels of IFNγ origin from NK cells activated vigorously by intercellular regulatory signals sent by T cells. Increased concentrations of this cytokine are harmful for pregnancy, causing fetal resorptions in the mice model of recurrent spontaneous abortions,[24] and could also be responsible for induction of trophoblast apoptosis.[25] Decreased IFNγ production by PBMCs was noted in pregnant women in remission of rheumatoid arthritis.[26] Of all investigated cytokines, only IFNγ is significantly increased when patients with transient hypertension are compared with normotensive pregnant controls. Despite the lack of changes concerning the rest of the investigated cytokines, this result indicates the presence of a prominent local Th1 shift[27,28] in transient hypertension similar to that existing in pre-eclampsia. The diminished production of IL-6 and IL-10 observed in our study indicates downregulation of Th2-type activity in decidual tissue, which is supplementary to that observed in peripheral blood[20] and placenta[29] during pre-eclamptic pregnancy, and may result in IFNγ overproduction. Deficiency in IL-6 secretion observed during pre-eclampsia lowers trophoblastic hCG production and causes defective placental development.[25] Lowered IL-10 production in pre-eclampsia could be associated with heightened maternal antifoetal immunity and inadequate placental development.[11] IL-12 promotes the potential of CD4 T cells to produce IFNγ; however, decreased IL-12 production does not result in inhibition of IFNγ secretion,[30] which is confirmed by our results.

Lymphocyte subset analysis indicates that the decidua of pre-eclamptic patients contains increased numbers of classical NK cells. This remains in agreement with results obtained by Stallmach *et al.*,[31] who found out that in pre-eclamptic patients NK $CD56^+$ cells constituted more than 40% of decidua infiltrating cells. The co-existence of a lowered $CD3^+$ T subset percentage together with NK population shift could be responsible for the observed cytokine disbalance.[32] Very low representation of decidual $CD19^+$ B lymphocytes in pre-eclampsia argues against thesis[33] of their possible role in pathogenesis of this syndrome. The classical NK $CD3^-/CD56^+16^+$ cells are more abundant in the decidua of healthy pregnant subjects then in pregnant women with transient hypertension. The candidate for local immunoregulatory mechanism inhibiting NK proliferation is antigen HLA-G, whose expression on the trophoblastic surface in the cases of transient hypertension is higher compared with pre-eclampsia.[34]

Taking into consideration the disturbances of local cytokine production by decidual lymphocytes '*in vitro*', we conclude that transient hypertension

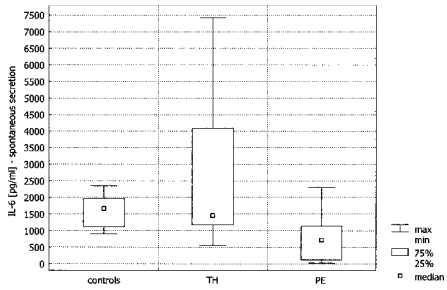

FIG. 1. Comparison of IL-6 spontaneous secretion in healthy pregnant women (controls) (n = 11), and pregnant women with transient hypertension (TH) (n = 17) and pre-eclampsia (PE) (n = 21). Statistically significant are the differences between controls and PE ($p < 0.002$) and between TH and PE ($p < 0.0008$).

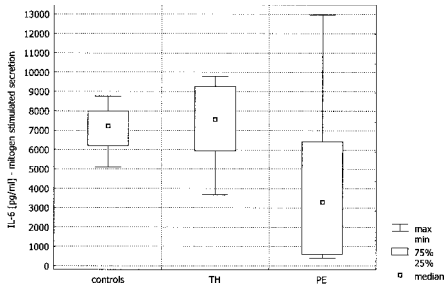

FIG. 2. Comparison of IL-6 PHA-stimulated secretion in healthy pregnant women (controls) (n = 11), and pregnant women with transient hypertension (TH) (n = 17) and pre-eclampsia (PE) (n = 21). Statistically significant are the differences between controls and PE ($p < 0.02$) and between TH and PE ($p < 0.002$).

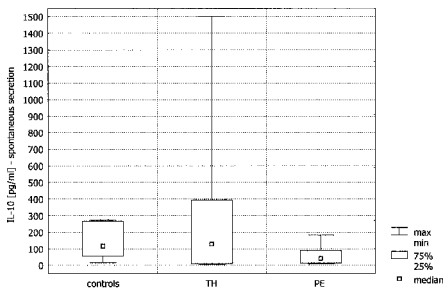

FIG. 3. Comparison of IL-10 spontaneous secretion in healthy pregnant women (controls) (n = 11), and pregnant women with transient hypertension (TH) (n = 17) and pre-eclampsia (PE) (n = 21). Statistically significant are the differences between controls and PE ($p < 0.05$).

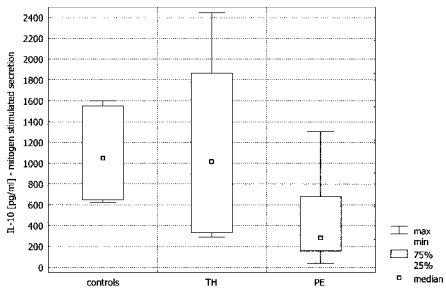

FIG. 4. Comparison of IL-10 PHA-stimulated secretion in healthy pregnant women (controls) (n = 11), and pregnant women with transient hypertension (TH) (n = 17) and pre-eclampsia (PE) (n = 21). Statistically significant are the differences between controls and PE ($p < 0.002$) and between TH and PE ($p < 0.008$).

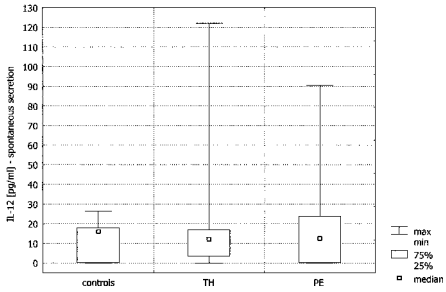

FIG. 5. Comparison of IL-12 spontaneous secretion in healthy pregnant women (controls) (n = 11), and pregnant women with transient hypertension (TH) (n = 17) and pre-eclampsia (PE) (n = 21). The differences between all groups studied are not statistically significant

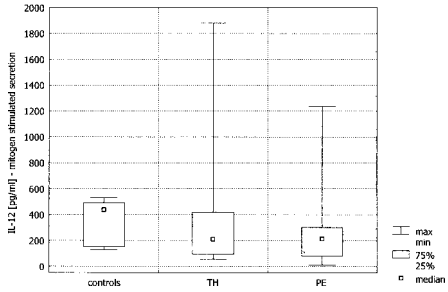

FIG. 6. Comparison of IL-12 PHA-stimulated secretion in healthy pregnant women (controls) (n = 11), and pregnant women with transient hypertension (TH) (n = 17) and pre-eclampsia (PE) (n = 21). Statistically significant are the differences between controls and PE ($p < 0.05$).

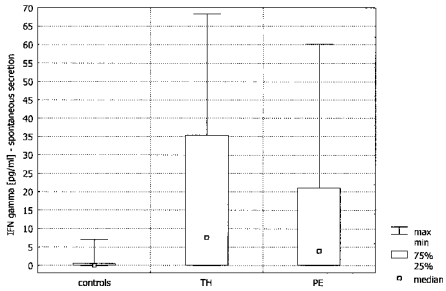

FIG. 7. Comparison of IFNγ spontaneous secretion in healthy pregnant women (controls) (n = 11), and pregnant women with transient hypertension (TH) (n = 17) and pre-eclampsia (PE) (n = 21). Statistically significant are controls and TH ($p < 0.03$).

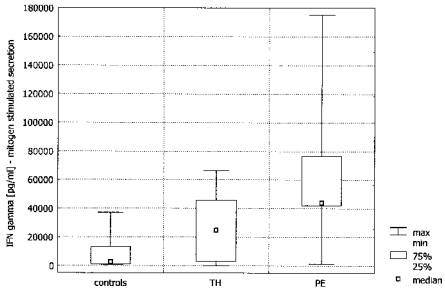

FIG. 8. Comparison of IFNγ PHA-timulated secretion in healthy pregnant women (controls) (n = 11), and pregnant women with transient hypertension (TH) (n = 17) and pre-eclampsia (PE) (n = 21). Statistically significant are the differences between controls and PE ($p < 0.0002$) and between TH and PE ($p < 0.02$).

constitutes an intermediate step in the etiology of pre-eclampsia and is like pre-eclampsia characterized by a disturbed Th1/Th2 balance, although intensity of these disturbances is different in each syndrome.

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