Comparison of early pregnancy serum concentration of neopterin, neopterin/creatinine ratio, C-reactive protein, and chitotriosidase, in pregnant women with birth at term and spontaneous preterm birth

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Abstract. Inflammatory mechanisms are involved in achieving a normal pregnancy and in the development of certain pregnancy complications. These changes are more intense in pregnant women that suffer of pregnancy complications, such as spontaneous preterm birth (SPB). This study compared the course of inflammatory markers (IM) [neopterin (Neo), neopterin/creatinine ratio (Neo/Cre), C-reactive protein (CRP), and chitotriosidase (Chito)] serum concentration in the early pregnancy of women with birth at term (BT) and preterm birth (PB). IM concentration was measured in 90 sera sampled from 45 pregnancies with BT and 30 sera from 15 pregnancies with PB. Two sera were sampled from each pregnant woman: one in the first trimester and another one in the second trimester. Early pregnancy IM concentration showed a direct correlation with gestational age: Neo (rho=0.262, P=0.004), Neo/Cre (rho=0.372, P<0.001), CRP (rho=0.187, P=0.041), and Chito (rho=0.039, P=0.66). The correlation was present in both categories of patients with BT and PB. Patients with PB before 34 week of pregnancy (wp) and 32 wp showed higher Neo and Neo/Cre concentration than BT patients. A significant association was found between the risk of PB before 34 wp, PB before 32 wp, and Neo concentration (PB <34 wp: odds ratio (OR) =5.13, P=0.035) (PB <32 wp: OR=8.2, P=0.020) and, respectively, Neo/Cre concentration (PB <34 wp: OR=5.29, P=0.015) (PB <32 wp: OR=9.25, P=0.006). No association between CRP or Chito and PB age was found. IM concentration correlates with the gestational age at the time of blood sampling. Increased Neo and Neo/Cre concentration are associated with PB. Further studies are needed to evaluate the usefulness of these markers in clinical practice.

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Abbreviations: IM, inflammatory markers; Neo, neopterin; CRP, C-reactive protein; Cre, creatinine; Chito, chitotriosidase; BT, birth at term; PB, preterm birth; SPB, spontaneous preterm birth; wp, week of pregnancy; OR, odds ratio; AOP, age of pregnancy

Key words: preterm birth, risk, early pregnancy, inflammatory markers, serum concentration

Introduction

Inflammatory markers (IM) are used to monitor the course of certain diseases such as infections, autoimmune diseases, cancer, graft rejection, post surgery monitoring or hemodialysis patients (1-4). In pregnant women studies have revealed

a mild increase of IM concentration and a weak activation of the cellular immune response during normal pregnancy (5,6). These changes are more intense in pregnant women that suffer of pregnancy complications, such as spontaneous preterm birth (SPB), spontaneous abortion, chorioamnionitis or preeclampsia (6-12). C-reactive protein (CRP), neopterin (Neo), and chitotriosidase (Chito) are the most studied IM in these situations (5,8).

Neo is a biomolecule synthesized by human macrophages consequent to stimulation with interferon-γ and is recognized as a marker of cellular immune system activation (13). Neo level is useful to monitor the progression of patients with graft rejection, autoimmune diseases, certain chronic diseases and infections (4,14-16). In graft recipients Neo levels increase a few days before symptoms of graft rejection complications appear (17). Also, graft recipients with a high post-surgery Neo level have a poor graft survival (18). Kidney function influences the Neo serum concentration because Neo is excreted without metabolisation via the kidney. This is why neopterin/creatinine ratio (Neo/Cre) better reflects inflammatory changes and monocyte/macrophage activation than Neo concentration alone (19).

Chito is a catabolic product of monocytes/macrophages used as a marker of macrophage activation and inflammation (20,21). Previous studies suggested that macrophages play a role in the processes of implantation and placentation (22) and in placental disorders, such as preeclampsia (23). A recent study found that pregnant women with threatened preterm labor who had preterm birth (PB) showed higher Chito serum concentrations than the ones who gave birth at term (BT) (8).

CRP is a protein that is increased in inflammation (24) and is synthesized in the liver upon stimulation with interleukin-6 (IL-6) released by macrophages and T cells (24). CRP is recognized as 'acute phase protein' (25). Previous studies found an association between CRP and PB, but the results were controversial (26). Keskin *et al* (8) did not find a difference between women with threatened PB and pregnant women with BT. In the practice CRP is used to monitor patients with premature rupture of membranes in order to capture the first biological signs of a chorioamnionitis (11,27).

Since the majority of studies analyzed the course of IM in late pregnancy (28) or at birth, only a few studies have focused on the course of IM in the first half of pregnancy. Inflammatory mechanisms seem to be involved in the achieving of a normal pregnancy and in the development of certain pregnancy complications. Because the mechanisms leading to pregnancy complications are often initiated before symptoms occur (5,29), the study of IM in early pregnancy could contribute to decoding the pathophysiological mechanisms involved in these situations and to opening the door to the development of new diagnostic tests and prevention strategies (30,31).

The worldwide incidence of PB is ~10% of all births and PB is one of the main contributors to fetal morbidity and mortality (32). Spontaneous and iatrogenic PB are different forms of PB. While iatrogenic preterm delivery is determined by a medical intervention for a maternal or fetal condition, spontaneous PB includes preterm labor, premature rupture of membranes and cervical weakness (30,31). In the clinical practice there is an acute demand to develop tests which identify pregnant women with risk of SPB at an early pregnancy age. Recognition of pregnant women at risk of premature birth could allow the implementation of preventive measures to minimize complications (31).

Starting from our previous research which revealed that Neo correlates with gestational age and predicts PB in asymptomatic pregnant women (5), we analyzed the course of other IMs (CRP, Chito, and Neo/Cre ratio) in sera consecutively collected early in pregnancy from women with BT and PB.

Patients and methods

Patients and sera. Two sera were sampled from each pregnant woman: one in the first trimester [4-13 weeks of pregnancy (wp)] and another one in the second trimester (15-22 wp). Sera were frozen at -80˚C. Medical files were analyzed retrospectively and the patients were classified according to the time of delivery (18).

Characterization of pregnant women with BT. Ninety sera were sampled from 45 pregnant women without pregnancy associated disorders and with BT: one sample in the first trimester between 4-13 wp and a second sample in the early second trimester between 15-22 wp.

Characterization of pregnant women with SPB. Thirty sera were sampled from 15 pregnant women with PB: one sample in the first trimester between 4-13 wp and a second sample in the early second trimester between 15-22 wp. Of the 15 pregnant women with PB ≤34 wp, 9 had a PB before ≤32 wp.

Detection of Neo concentration. The Neo concentration was measured using standard test kits (Neopterin EIA; Thermo Fisher Scientific, Inc.). Values are expressed in nanomoles per liter (nmol/l).

Detection of CRP and CRE concentration in sera. The CRP, concentration and CRE concentration was measured using standard test kits (FUJI DRI-Chem Slide CRP-SIII and FUJI DRI-Chem Slide CRE-SIII; Fuji Film Corporation and Nishiazabu 2-Chrome, respectively). Values are expressed in milligrams/liter (mg/l) (CRP) and milligrams per deciliter (mg/dl) (CRE).

Measurement of Chito activity in sera. Chito activity was measured by incubating 5 µl of serum with 100 µl of 0.022 mM 4-methylumbelliferyl-β-D-N,N',N''-triacetyl chitotriose (4-MU-chitotrioside; Sigma-Aldrich; Merck KGaA) as substrate in citrate/phosphate buffer (0.1/0.2 M), pH 5.2, at 37˚C for 60 min. The reaction was stopped with 120 µl of 0.5 M $NaHCO_3/Na_2CO_3$ buffer, pH 10.7. Fluorescent 4-methylumbelliferone (4-MU) was measured with a fluorimeter (Infinite 200 PRO, Tecan Deutschland GmbH) with excitation at 360 nm and emission at 465 nm. The activity of Chito was calculated by using a standard curve of 4-MU with 12 different concentrations from 0-10 µM. Activities are expressed in nanomoles per milliliter per hour (nmol/ml/h).

Study design and statistical analysis. Patients were enrolled in this cohort-retrospective, non-interventional study according

Table I. Comparison of inflammatory marker concentration between the studied sub-groups.

Variables	BT (Group A)	PB <34 wp (Group B)	PB <32 wp (Group C)	P-value (A vs. B vs. C)	P-value (A vs. B)	P-value (A vs. C)
AOP (days)	120 (7)	119 (12)	118 (10)	0.455	0.882	0.195
Neo (nmol/l)	6.16 (1.99)	7.04 (4.58)	8.92 (7.39)	0.046[a]	0.126	0.042[a]
CRP (mg/l)	4.0 (5.0)	3.0 (3.0)	4.0 (3.0)	0.853	0.640	0.693
Neo/Cre	7.53 (2.77)	9.39 (5.41)	12.10 (7.75)	0.008[a]	0.031[a]	0.009[a]
Chito (nmol/l)	27.3 (23.3)	24.0 (13.6)	26.4 (29.6)	0.890	0.807	0.670

Results are presented as median and interquartile range. [a]Differences between groups are significant at α=0.05 threshold. wp, week of pregnancy; BT, birth at term; PB, preterm birth; AOP, age of pregnancy; Neo, neopterin; CRP, C-reactive protein; Neo/Cre, neopterin/creatinine; Chito, chitotriosidase.

to a consecutive-case population base. Data were collected and analyzed using the SPSS v.17 software suite (SPSS Inc.) and since the variables were non-Gaussian distributed the results are presented as median and (interquartile range) for continuous variables, respectively, percentages and number of individuals for categorical variables. To assess the significance of the differences between groups Mann-Whitney-U test and Kruskal-Wallis (medians, non-Gaussian populations), respectively, Fisher's exact tests (proportions) were used. Continuous variable distributions were tested for normality using Shapiro-Wilk test, and for equality of variances using Levene's test.

The strength of the association between two continuous variables from non-Gaussian populations was evaluated using Spearman's correlation coefficient. Its statistical significance was assessed using t-score distribution test. Risk was assessed using the odds ratio (OR) value for dichotomous variables, respectively, by building logistic regression models for continuous variables. In these models the exponent of B was assimilated to the OR increase and is expressed for each increase with one unit in the studied parameter, in relation to the previous odds.

We did not find any statistically significant difference in gestational age at the time of sampling the sera in the studied groups: term vs. preterm, term vs. preterm ≤34 wp or term vs preterm ≤32 wp. This is why the gestational age at sampling does not significantly influence the comparison between the analysis of groups as a whole and the analysis of groups according to trimesters.

Ethical considerations. The present study meets the ethical guidelines, including adherence to the legal requirements of the study country. Informed consent was obtained from each patient. The study was approved by the Institutional Board of the 'Victor Babes' University of Medicine and Pharmacy (Timisoara, Romania) (approval no. 848/06.04.2011).

Results

Correlation between age of pregnancy (AOP) and IM (Neo, Neo/Cre ratio, CRP, and Chito) concentration in early pregnancy serum of women with BT and PB. We correlated AOP at the time of blood sampling (expressed in days from the last menstrual period) with IM (Neo, CRP, Chito and Neo/Cre ratio) concentration measured in 120 sera collected in the first and second trimester from 60 pregnant women. There were 90 sera collected from 45 pregnant women with BT and 30 sera from pregnant women with PB ≤34 wp, of which 18 sera were from 9 pregnant women with PB ≤32 wp. A significant correlation between AOP and Neo was present in all pregnant women (rho=0.262, P=0.004), pregnant women with BT (rho=0.204, P=0.053) and PB (rho=0.392, P=0.032), Neo/Cre ratio showed a better correlation with AOP than Neo in all pregnant women (rho=0.372, P<0.001), pregnant women with BT (rho=0.317, P=0.002) and PB (rho=0.504, P=0.004). CRP correlated with AOP in all pregnant women (rho=0.188, P=0.041), in the BT group (rho=0.192, P=0.069), but not in the PB group (rho=0.162, P=0.392). No correlation was found in early pregnancy between gestational age and Chito sera concentration in the three categories of pregnant women: all (rho=0.039, P=0.660), BT (rho=0.093, P=0.383), and PB (rho=-0.211, P=0.262).

Second trimester sera of IM concentration (Neo, Neo/Cre ratio, CRP and Chito) in pregnant women with BT, PB before 34 wp and PB before 32 wp. AOP at the time of blood sampling showed no significant difference between pregnant women with BT and PB ≤34 wp (120 vs. 119 days; P=0.822), respectively, PB ≤32 wp (120 vs. 118 days; P=0.195). Both categories of pregnant women with PB ≤34 wp and PB ≤32 wp showed second trimester sera Neo concentrations higher than pregnant women with BT: (7.04 vs. 6.16; P=0.126), respectively (8.92 vs. 6.16; P=0.042), however, the significance threshold was reached only for the variation of medians between the three groups, respectively, between PB≤32 wp vs. BT, but not for PB ≤34 vp vs. BT. PB was associated with an increased Neo/Cre ratio, increase which was valid for both PB ≤34 wp vs. BT (9.39 vs. 7.53; P=0.031) and PB≤32 wp vs. BT (12.10 vs. 7.53; P=0.009). No significant differences were observed regarding the CRP and Chito between the three groups or between pairs of two separately studied sub-groups. The detailed comparison of the studied parameters, stratified by the outcome of the birth status is presented in Table I.

Association between IM (Neo, Neo/Cre ratio, CRP and Chito) concentration in second trimester sera and gestational age at

birth. Of the 45 pregnant women with BT, 4 women had a Neo concentration of >9 nmol/l while in the SPB group≤34 wp 5 out of 15 (OR=5.13; P=0.035) and in the SPB group ≤32 wp 5 out of 9 pregnant women showed a Neo concentration >9 nmol/l (OR=8.2; P=0.020). Neo/Cre ratio was shown to be better associated to PB than Neo. Eight out of 45 pregnant women with BT and 8 out of 15 pregnant women with SPB ≤34 wp (OR=5.29; P=0.015), respectively, 6 from 9 pregnant women with SPB ≤32 wp (OR=9.25, P=0.006) showed a Neo/Cre ratio concentration of >9.3. Since CRP and Chito showed similar concentrations in the three categories of pregnant women (BT, PB ≤34 and PB ≤32 wp), no association between PB and CRP, respectively, or Chito concentration in sera of second trimester pregnant women was found.

To evaluate the impact of the increase in the values of IM (considered as continuous variables) on the outcome of the pregnancy (considered as a dichotomous variable) we built univariate logistic regression models having as a dependent variable the PB, respectively, independent variables the serum IM levels. According to these models, we observed that increases in the levels of Neo and Neo/Cre ratio were associated with a significant increase of the odds for PB: for each increase with 1 nmol/l in Neo value the odds for PB increases with 32.1% compared with the previous odds (OR=1.321; P=0.048), respectively, for each increase with 1 unit in the Neo/Cre ratio the odds for PB increases with 22.2% (OR=1.222; P=0.041). No significant associations were found between the odds for developing PB and CRP values (OR=1.005; P=0.818) or Chito values (OR=1.000; P=0.982).

Discussion

This is the first study comparing the course of IM (Neo, Neo/Cre ratio, CRP and Chito) concentration in the first half of pregnancy of pregnant women with BT and PB and analyzes the association between PB and these IM concentrations in sera sampled 8-12 weeks before PB occurs. Our study adds to other studies that analyze IM in late pregnancy or at the time of threatened preterm labour and birth (7-11) and confirms that normal pregnancy is associated with a weak activation of the cellular immune system and with a more intense immune activation in pregnant women with PB (5,8). The strongest correlation of gestational age was found with Neo/Cre ratio, succeeded by Neo and CRP concentration. Chito did not show any correlation with gestational age, while in the PB group CRP correlated with the gestational age, but the correlation did not reach a significant threshold. We do not have an explanation as to why CRP does not correlate with GA in PB patients. We suppose that since the correlation between GA and CRP was weaker and the number of cases smaller in this group, the correlation was not sufficiently strong to reach a significant value.

Our results showed that in early pregnancy Neo better correlates with gestational age and it is a more precise marker of inflammatory changes than CRP. Since Neo is excreted without metabolisation via kidney we expected that Neo/Cre ratio may be a better marker of inflammatory changes than Neo alone (19). Our results confirm this assumption and are in concordance with observations in graft recipients where Neo/Cre ratio had a better power of prediction of graft survival than Neo alone (19).

CRP is an IM produced by the liver upon stimulation with IL-6 while Neo is produced by monocytes/macrophages upon stimulation with interferon-γ. Monocytes/macrophages are important players at the materno-fetal interface and have an implication in implantation, tissue configuration and placental disorders (8,22,23). Therefore changes at the materno-fetal interface could involve monocyte/macrophage activation and release of Neo. Also, monocytes/macrophages are involved in the initiation and maintenance of inflammation. It is known that inflammatory changes at the level of the myometrium could determine myometrium contraction and preterm labour (8,33-36). Infectious and non-infectious factors could trigger inflammation mechanisms at the materno-fetal interface (37). Lee *et al* (38-40) suggested that activation of the maternal immune system could play an important role in the pathogenesis of premature birth and that in certain situations the immunological reaction could be compared with a materno-fetal rejection. Immunological changes begin before clinical symptoms occur. Relying on sera collected in early pregnancy we have the opportunity to evaluate the IM concentration in pregnant women with BT and PB, 8-12 weeks before PB occurs (41). Our results reveal that only Neo and Neo/Cre ratio, but not CRP and Chito showed elevated concentrations in second trimester sera of pregnant women with PB ≤34 wp and ≤32 wp compared with those with BT.

Neo/Cre ratio and Neo concentrations in early second trimester showed the best association with PB whereas CRP and Chito concentrations showed no association. The results are in line with our previous studies that showed that early pregnancy Neo serum concentration predicts PB (5) and with other studies that showed CRP is not elevated in pregnant women with threatened preterm labor (8). Interestingly, while the study of Keskin *et al* (8) showed only a small increase of Chito concentration in pregnant women with threatened preterm labour compared with pregnant women with BT, our results showed no difference between the early second trimester sera Chito concentration in pregnant women with PB and BT. An explanation could be that the Chito concentration increases only at the time of, or shortly, before the threatened preterm labor.

Increased Neo values do not automatically imply immunological changes at the materno-fetal interface and must be considered as a sign of inflammation in the body. Complementary investigations should be undertaken to clarify the differential diagnosis. Such an approach may be solved in prospective studies that should evaluate the predictive value of Neo and Neo/Cre ratio in carefully selected cohorts of patients according to the etiology of PB.

In conclusion, our study provides evidence that in early pregnancy gestational age correlates with an increase in IM concentration of Neo, CRP and Neo/Cre ratio, but not of Chito. Only increased Neo and Neo/Cre ratio are associated with an increased risk of PB.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DBN, DLS and PT wrote the manuscript. BT and ZC performed the statistical analysis of the data and contributed in the writing of the manuscript. DBN and REB performed the experiments. FB was responsible for the chitriosidase determination. MLC, RV, DN, REB and OC drafted the manuscript and revised it critically for important intellectual content. DN, DLS, PT, MLC, OC and RV were involved in the conception of the study and data interpretation. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study meets the ethical guidelines, including adherence to the legal requirements of the study country. Informed consent was obtained from each patient. The study was approved by the Institutional Board of the 'Victor Babes' University of Medicine and Pharmacy (Timisoara, Romania) (approval no. 848/06.04.2011).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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