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Association of osteocalcin, insulin resistance and oxidative stress during noncomplicated pregnancy

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Abstract

Background: The aim of this study was to explore longitudinal changes of serum osteocalcin during normal, uncomplicated pregnancy and after delivery, and its correlations with parameters of glucose homeostasis, lipid status, and oxidative status in late pregnancy.
Methods: Osteocalcin, glucose, insulin, lipid status parameters, total oxidative status (TOS), and total antioxidant capacity (TAC) were measured in sera of 38 healthy pregnant women. The sera were collected at the midpoint of the 1st, in the 2nd and 3rd trimester, and after delivery. Homeostatic model assessment (HOMA) indices were calculated and used as surrogate markers of insulin resistance.
Results: Repeated measures analysis of variance showed a progressive increase in total cholesterol, triglycerides, and low density lipoprotein (LDL)-cholesterol, with a postpartum decrease. High density lipoprotein (HDL)-cholesterol increased in the 2nd trimester and decreased after delivery. Total oxidative status (TOS) increased significantly in the 3rd trimester ($p<0.001$). TAC showed a significant increase after delivery ($p<0.05$). Insulin showed a significant increase in the 3rd trimester ($p<0.05$). Homeostatic model assessment (HOMA)-%B increased significantly in the 3rd trimester ($p<0.001$). Osteocalcin showed a decrease in the 2nd trimester, and a marked increase in the 3rd trimester and postpartum ($p<0.001$). Osteocalcin was significantly positively correlated with BMI, insulin, HOMA of insulin resistance (HOMA-IR), HOMA-%B, TAC ($p<0.05$), triglycerides and uric acid ($p<0.001$). Multiple regression analysis showed that TAC is independently associated with osteocalcin level during 3rd trimester ($p<0.05$).
Conclusions: We observed the changes in pregnancy that may lead towards atherogenic, prooxidant and insulin resistant state, which are possibly counterbalanced by various protective systems, one of which might be osteocalcin.

Keywords: insulin resistance; osteocalcin; oxidative stress; pregnancy.

Introduction

It is well known that insulin resistance in late pregnancy is the after effect of the physiological adaptation necessary to provide glucose to the growing fetus. It is thought to be a consequence of combination of increased maternal adiposity and insulin-desensitizing effects of the hormones of the placenta. The most important effects of estrogen, progesterone, and human placental lactogen are proven by an increase of glucose concentrations and a compensatory increase in insulin secretion in order to maintain normoglycemia in mothers [1]. Those who fail to produce enough insulin, or have inadequate β-cell compensation for the body's insulin needs, develop gestational diabetes mellitus (GDM) [2].

Pregnancy is accompanied by a high energy and oxygen demand and it is characterized by susceptibility to oxidative stress [3]. Oxidative stress occurs when the production of prooxidants overcomes the antioxidative capacity. Prooxidant species are thought to have a physiological role, but when they are produced in excess, they might be implicated in the development of pregnancy-related disorders, such as GDM and preeclampsia [4].

Results of previous studies indicate an important influence of bone-derived protein, osteocalcin, on pancreatic production of insulin [5, 6]. Also, osteocalcin showed impact on insulin sensitivity of the peripheral tissue and it is suggested to be an endocrine link between glucose

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metabolism and bones [5, 6]. This association has been well documented in different pathologies, but only a few studies investigated the link between osteocalcin and insulin resistance during normal, uncomplicated pregnancy [7, 8]. Furthermore, osteocalcin is used in clinical practice as a marker of bone formation and bone turnover. As pregnancy is a state of altered bone turnover, its concentration in blood changes throughout pregnancy [9, 10]. The aim of this study was to explore longitudinal changes of serum osteocalcin during normal, uncomplicated pregnancy and after delivery, and its correlations with parameters of glucose homeostasis, lipid status and oxidative status in late pregnancy.

Materials and methods

For this longitudinal study, women were recruited at their first prenatal visit to the laboratory of Poliklinika Medilab, Čačak, Serbia. The exclusion criteria were non-singleton pregnancy or development of any pregnancy-related complication. All women underwent oral glucose tolerance test (OGTT) with 75 g of glucose between weeks 24 and 28, and showed normal glucose tolerance according to National Guidelines for Diabetes Mellitus [11]. A full medical history was taken from each participant, including preexisting disorders, smoking status and nutritional habits, by the trained staff. They were all non-smokers and were given advice on balanced diet, which included six to 11 servings of bread and grains, two to four servings of fruit and three servings of protein sources daily. All women used vitamin supplementation which contained approximately 400 μg of folic acid, 400 IU of vitamin D, 200–300 mg of calcium, 70 mg of vitamin C, 3 mg of thiamine, 2 mg of riboflavin, 20 mg of niacin, 6 μg of vitamin B12, 10 mg of vitamin E, 15 mg of zinc and 17 mg of iron.

In total, 38 women participated in the study. The median of age was 30 (range 20–37). All of them had uneventful pregnancies and term delivery. All infants were healthy and appropriate in size for gestational age (they had birth weight between 10^{th} and 90^{th} percentiles).

The study was planned and conducted according to the ethical standards of the Declaration of Helsinki and according to local institutional guidelines. The local Institutional Review Committee approved the research proposal. Informed consent was obtained from all individuals involved in the study.

Body-mass index (BMI) was calculated as weight (kg)/squared height (m^2).

The samples were taken at the midpoint of the first trimester (T1), in second (T2), third trimester (T3) and more than 4 weeks postpartum (T4), after an overnight fast (>10 h), between 7 am and 9 am, in a serum test tube. The samples were centrifuged and for the delayed analyses, the sera were aliquoted and stored at −80 °C within 1 h of sampling. Thereafter, the samples were thawed only once, and that was immediately before analysis. The measurements of glucose, total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) cholesterol and uric acid were performed immediately.

Glucose, TC, TG, HDL cholesterol, and uric acid were measured using commercial kits on Roche Cobas c311 clinical chemistry analyzer (Roche Diagnostics, Mannheim, Germany). Insulin, N-MID osteocalcin and 25-hydroxy vitamin D [25(OH)D] were measured by electrochemiluminescence immunoassay (ECLIA) on the Cobas e411 analyzer (Roche Diagnostics, Mannheim, Germany). The intra- and inter-assay coefficients of variance were as follows: for insulin 2.0% and 3.9%, respectively, for N-MID osteocalcin 2.0% and 4.1%, respectively, and for 25(OH)D 2.7% and 5.2%, respectively. For each, number of measurements was 10, during 10 days, using same lot of reagents for all of the measurements.

Roche N-MID osteocalcin assay detects stable N-MID-fragment as well as the intact osteocalcin, and it remains unchanged after 3 h at room temperature and after 24 h at 4 °C [12].

Low density lipoprotein (LDL) cholesterol and homeostatic model assessment (HOMA) of insulin resistance and β cell function (HOMA-IR and HOMA-%B) indices were calculated using following formulas:

LDL cholesterol (mmol/L)=TC (mmol/L)−HDL cholesterol (mmol/L) −TG (mmol/L)/2.22 (Friedewald equation) [13]

$$\text{HOMA-IR}=(\text{glucose}\times\text{insulin})/22.5$$

$$\text{HOMA-\%B}=(20\times\text{insulin})/(\text{glucose}-3.5),$$

where glucose is expressed in mmol/L and insulin in mU/L (14).

The Friedewald equation is acceptably accurate compared to the direct measurement of LDL-cholesterol, except in samples that have triglyceride concentrations above 4.52 mmol/L or those that contain increased quantities of chylomicrons, like in nonfasting specimens [15].

Total oxidative capacity (TOS) was measured according to Erel's method [16], applied to the Ilab 300 Plus autoanalyzer (Instrumentation Laboratory, Milan, Italy). The method is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in the serum and measurement of ferric ion using xylenol orange. The assay was calibrated with hydrogen peroxide (H_2O_2) and the results were expressed in terms of micromolar H_2O_2 equivalent per liter (μmol H_2O_2 equiv/L). The intra-assay and inter-assay coefficients of variance were 5.6% and 9.5%, respectively. For the calculation of intra-assay coefficient of variance the number of measurements was 10, and for the calculation of inter-assay coefficient of variance we used 10 samples over 10 days.

Total antioxidant capacity (TAC) was determined according to Erel's method [17], which was also applied to Ilab 300 Plus autoanalyzer. It is based on decoloration of 2.2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS) by antioxidants in serum. The assay was calibrated with Trolox (a water-soluble analog of vitamin E, 6-xydroxy-2.5.7.8-tetramethylchroman-2-carboxylic acid). The results were expressed as mmol Trolox equivalent/L. The intra- and inter-assay coefficients of variance were 4.3% and 8.8%, respectively. For the calculation of intra-assay coefficient of variance the number of measurements was 10, and for the calculation of inter-assay coefficient of variance we used 10 samples over 10 days.

Statistical analysis

Data are shown as the arithmetic mean±standard deviation (SD) with range in parenthesis for normally distributed variables.

Log-transformed variables are shown as the geometric mean and the 95th confidence intervals [18]. A comparison of continuous variables was performed by repeated measures analysis of variance with post hoc Bonferroni correction. We used multiple regression analysis to estimate the independent association of the investigated parameters with osteocalcin concentration in late pregnancy (3rd trimester). Spearman's rho correlation test was used for screening the independent variables. If p-values were <0.10, the variables were included in further regression analysis. The tolerance option was used to prevent multicollinearity among the independent variables [19]. Statistical analysis was performed using MedCalc Software (Ostend, Belgium).

Results

General characteristics and biochemical parameters of the study group are shown in Table 1. Statistically significant p-values are bold in Tables 1–4. As was expected, BMI increased during pregnancy. Uric acid increased progressively during pregnancy and continued to increase after delivery (Table 1). TC and TG levels showed a similar progressive increase over pregnancy, with a postpartum decline, where the postpartum TC level was still significantly higher than the 1st trimester value, and the postpartum TG value was somewhat similar to the 1st trimester value. HDL-cholesterol increased in the 2nd trimester in comparison to the 1st trimester and dropped after delivery to the value lower than all of the pregnancy values. Comparably to TC, LDL-cholesterol increased during pregnancy. After delivery, its value remained higher than the 1st and 2nd trimester values (Table 1).

TOS level showed no change in the 2nd trimester, but it increased significantly in the 3rd trimester and then decreased after delivery to a value close to the values of the first two trimesters. TAC levels only showed significant increase after delivery in comparison to the 2nd trimester value. There was a slight increase in the 3rd trimester, but it did not reach statistical significance (Table 1).

All of the participants retained normal glucose level throughout pregnancy, and there were no changes in its concentration until after delivery, at which point it was significantly higher compared to the 2nd trimester value (Table 2). Insulin levels showed a significant increase in the 3rd trimester, as expected, owing to the increased insulin resistance in the late pregnancy. HOMA-IR showed a notable, albeit not statistically significant, rise in the 3rd trimester, but the postpartum value was significantly lower compared to the 3rd trimester. HOMA-%B increased significantly in the 3rd trimester and then dropped to a value significantly lower than the pregnancy values (Table 2).

25(OH)D levels showed a significant rise in the 2nd trimester and no change in the 3rd trimester, and a significant decrease after delivery. Osteocalcin showed a decrease in the 2nd trimester, but it increased markedly in the 3rd trimester and even more strikingly post partum (Table 2).

Spearman's correlation analyses were performed to test for associations between osteocalcin and other investigated parameters (Table 3). Osteocalcin was significantly positively correlated with BMI, TG, uric acid, insulin, HOMA-IR, HOMA-%B, and TAC.

In order to find which of investigated parameters was independently associated with significant increase of osteocalcin in the 3rd trimester, we performed multiple regression analysis. To prevent multicollinearity we excluded insulin concentration and HOMA-%B from the primary model. The result of this analysis showed that TAC is independently associated with osteocalcin level during 3rd trimester (Table 4).

Table 1: Clinical and laboratory parameters in women during pregnancy.

	1st Trimester n=38	2nd Trimester n=38	3rd Trimester n=38	After delivery n=38	p-Value
Week of gestation	12.9±0.65	23.1±0.52	31.7±0.12	4.7±0.43	
BMI, kg/m^2	22.3±1.2 (17.1–28.8)	23.4±1.1 (18.0–31.1)[a,e]	26.5±1.1 (19.9–32.6)[a,b,e]	23.9±1.1 (17.0–30.5)[a,c,e]	**<0.001**
Uric acid, µmol/L	173.9±11.5 (107–257)	187.4±12.8 (112–292)[a,e]	239.1±14.5 (144–334)[a,b,e]	262.4±16.2 (155–383)[a,b,e]	**<0.001**
t-C, mmol/L	4.6±0.2 (3.5–6.1)	5.3±0.2 (4.0–6.8)[a,e]	6.6±0.3 (4.7–9.2)[a,b,e]	5.2±0.3 (3.2–7.5)[a,c,e,f]	**<0.001**
TG, mmol/L[d]	0.99 (0.85–1.15)	1.37 (1.19–1.59)[a,e]	2.74 (2.41–3.11)[a,b,e]	0.96 (0.80–1.17)[b,c,e,f]	**<0.001**
HDL-C, mmol/L	1.8±0.1 (0.9–2.7)	1.9±0.1 (1.0–2.6)[a,f]	1.8±0.2 (1.1–3.2)	1.5±0.1 (0.8–2.3)[a,b,c,e]	**<0.001**
LDL-C, mmol/L	2.3±0.2 (1.4–3.8)	2.7±0.2 (1.4–4.0)[a,e]	3.5±0.3 (1.0–5.5)[a,b,e]	3.1±0.3 (1.7–5.5)[a,b,e,f]	**<0.001**
TOS, µmol/L[d]	10.3 (8.6–12.3)	10.7 (9.1–12.5)	16.5 (13.9–19.7)[a,b,f]	10.6 (8.5–13.4)[c,f]	**<0.001**
TAC, mmol/L[d]	0.67 (0.62–0.73)	0.67 (0.60–0.74)	0.73 (0.67–0.78)	0.76 (0.69–0.84)[b,f]	**<0.05**

Pairwise comparison; [a]mean difference significantly different from the 1st trimester; [b]mean difference significantly different from the 2nd trimester; [c]mean difference significantly different from the 3rd trimester. Data are expressed as arithmetic mean±standard deviation (SD) with the range in parenthesis for normally distributed variables. [d]Log-transformed variables are shown as geometric mean and the 95th confidence intervals. [e]p<0.001 (Bonferroni corrected), [f]p<0.05 (Bonferroni corrected). t-C, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TOS, total oxidative status; TAC, total antioxidative capacity.

Table 2: Glucose concentrations, insulin concentrations, vitamin D and osteocalcin concentrations during pregnancy.

Parameter	1st Trimester n=38	2nd Trimester n=38	3rd Trimester n=38	After delivery n=38	p-Value
Week of gestation	12.9±0.65	23.1±0.52	31.7±0.12	4.7±0.43	
Glucose, mmol/L	4.7±0.1 (3.8–5.2)	4.5±0.1 (3.7–5.2)	4.8±0.2 (3.5–5.3)	4.8±0.2 (3.9–5.8)[b,f]	0.05
Insulin, μU/mL	10.3±2.4 (2.7–36.9)	9.6±1.9 (2.2–27.6)	16.4±5.0 (4.6–74.2)[b,f]	6.9±1.4 (2.2–23.2)[a,c,f]	**<0.05**
HOMA-IR	2.23±0.58 (0.48–8.20)	1.98±0.42 (0.39–5.88)	3.73±1.4 (0.86–16.8)	1.50±0.32 (0.44–5.79)[c,f]	**<0.001**
HOMA-%B[d]	149.6 (122.7–182.4)	170.7 (142.3–204.8)	222.8 (188.2–263.8)[a,b,f]	97.9 (82.6–116.0)[a,b,c,e,f]	**<0.001**
25(OH)D, ng/mL[d]	15.3 (13.1–17.9)	19.0 (16.7–21.6)[a,e]	16.8 (14.4–19.6)	12.9 (10.9–15.3)[b,c,e,f]	**<0.001**
Osteocalcin, ng/mL	13.9±1.5 (6.9–22.6)	11.7±1.0 (6.0–19.1)[a,f]	17.3±1.9 (8.0–31.9)[a,b,e,f]	35.4±3.3 (13.3–58.6)[a,b,c,e]	**<0.001**

Pairwise comparison; [a]mean difference significantly different from the 1st trimester; [b]mean difference significantly different from the 2nd trimester; [c]mean difference significantly different from the 3rd trimester. Data are expressed as arithmetic mean±standard deviation (SD) with range in parenthesis for normally distributed variables. [d]Log-transformed variables are shown as the geometric mean and the 95th confidence intervals. [e]$p<0.001$ (Bonferroni corrected), [f]$p<0.05$ (Bonferroni corrected). HOMA-IR and%B, homeostatic model assessment of insulin resistance and β cell function; 25(OH)D – 25-hydroxy vitamin D.

Table 3: Spearman's non-parametric correlations between osteocalcin concentration and biochemical and oxidative status parameters in 3rd trimester of pregnancy.

Parameter	ρ	p-Value
BMI, kg/m²	0.293	**<0.05**
Glucose, mmol/L	0.161	0.164
T-C, mmol/L	0.179	0.121
TG, mmol/L[a]	0.314	**<0.001**
HDL-C, mmol/L	–0.103	0.377
LDL-C, mmol/L	0.104	0.370
Uric acid, μmol/L	0.382	**<0.001**
Insulin, μIU/L	0.323	**<0.05**
HOMA-IR	0.310	**<0.05**
HOMA-%B	0.374	**<0.05**
25(OH)D, ng/mL	0.079	0.501
TAS, mmol/L	0.324	**<0.05**
TOS, μmol/L	–0.039	0.742

Table 4: Multiple regression analysis for the association of investigated parameters with the osteocalcin concentration.

	Osteocalcin R^2=0.376 Adjusted R^2=0.172		
	β	SE (β)	p-Value
BMI, kg/m²	0.198	0.184	0.081
HOMA-IR	0.158	0.204	0.159
TAS, mmol/L	0.228	4.515	**<0.05**

Discussion

Observed changes in TC, TG, HDL- and LDL-cholesterol are in line with previous studies [20, 21]. After an initial decrease in the concentrations of lipids (during the first 8 weeks of pregnancy) [20], there is a steady increase until the delivery. In early pregnancy, increased estrogen, progesterone, and insulin favor lipid deposition and inhibit lipolysis, whereas in late gestation, rising concentrations of human chorionic somatomammotropin, prolactin, cortisol, and glucagon have antiinsulinogenic and lipolytic effects which lead to lipolysis and fat mobilization. These changes promote the use of lipids as a maternal energy source while preserving glucose and amino acids for the fetus [20]. Thus, hypertriglyceridemia develops in the late pregnancy.

Previous studies using the hyperinsulinemic-euglycemic glucose clamp technique [22] and mathematically derived OGTT-based indices [23] showed a decrease in insulin sensitivity during pregnancy. Although noteworthy, the change of HOMA-IR value in our study did not reach statistical significance until after delivery, when it was significantly lower compared to the value in the 3rd trimester, suggesting that the value in the late pregnancy might indeed be significantly higher than the pre-pregnancy value. HOMA-%B, however, showed a significant increase in the 3rd trimester, and then decreased markedly to a level lower than values at any time point during pregnancy, confirming that β-cell secretion increases during pregnancy so that women remain normoglycemic. Cellular mechanisms of these changes could be explained by an adaptive increase in the number of islets of Langerhans [24].

Osteocalcin concentrations showed a biphasic pattern, with a decrease in the 2nd trimester, followed by a significant increase in the 3rd trimester and postpartum, which was in concordance with the study of Ulrich et al. [9]. A similar biphasic pattern was observed in other studies [25, 26]. The decrease in mid-pregnancy may be explained by increased renal clearance and hemodilution [27]. The observed increase in the osteocalcin level

in the late pregnancy and especially during the postpartum period is a consequence of increased bone turnover, predominantly bone formation that follows the period of increased bone resorption in the 1st and 2nd trimester [9, 10]. Moreover, osteocalcin also has extra-skeletal effects and links bone to energy metabolism, which was first proven on animal and cell-based models [5, 6]. In humans, osteocalcin was found to be decreased in patients with DM2 [28], and negatively correlated with fasting plasma glucose, glycated hemoglobin [29, 30], HOMA-IR, BMI [31] and TG [31, 32] supporting the notion that the bone contributes to the regulation of glucose and lipid metabolism in humans with osteocalcin acting as a hormone that stimulates insulin sensitivity, insulin secretion, and energy expenditure.

In our study, osteocalcin also showed correlations with insulin, HOMA-IR, HOMA-%B, BMI, TG, uric acid, and TAC, but all of these correlations were positive. As all of the participants were healthy and had uneventful pregnancies, we might explain the correlations with insulin, HOMA-IR and HOMA-%B as a potential compensatory mechanism, which develops along with growing insulin resistance. This was previously suggested by Winhofer et al. [7], who showed that osteocalcin was higher in a group of women with GDM than in a group with normal glucose tolerance. Also, they proved that osteocalcin was in positive correlation with insulin secretion parameters. Similarly, another study [8] found a higher level of osteocalcin in women with GDM, but they found that maternal osteocalcin in early pregnancy did not predict the development of GDM.

On the other hand, increased insulin secretion during pregnancy might have an anabolic effect on bone metabolism, with insulin acting directly on bone cells and synergistically with other anabolic agents in the bone, such as insulin-like growth factor I and parathyroid hormone [33], thus contributing to the increased level of osteocalcin. Osteocalcin and insulin, therefore, might increase each other's activity or secretion in a feed forward loop.

Vitamin D has a role in calcium uptake and bone metabolism and also has a range of other, nonclassical actions, and its deficiency has been linked to the development of gestational diabetes, preeclampsia and neonatal morbidities [34]. The active metabolite 1,25-dihydroxyvitamin D, increases progressively in pregnancy, reaching its peak in the 3rd trimester [34]. The changes we found in 25(OH)D during pregnancy might be partly explained by the seasons of sample collection. All of the participants were recruited from April through June, so all of the 2nd trimester values were obtained during the summer season, and they were indeed significantly higher than the 1st trimester values. The last sampling was performed during the winter months, and that could explain the significantly lower levels of 25(OH)D at that time point. Vitamin D has a stimulatory effect on osteocalcin production [35], but we failed to show a significant correlation with osteocalcin concentration.

Uric acid is one component of serum TAC (33.1%), and the others are free sulfhydryl groups of serum proteins (52.9%), vitamin C (4.7%) and vitamin E (10%) [17]. Unlike some other studies [3, 36], our study failed to show a statistically significant increase on TAC in late pregnancy, but confirmed its increase after delivery. The result of multiple regression analysis showed that only TAC concentration is independently associated with osteocalcin level during the 3rd trimester. This association could be regarded in the light of the well-established relationship between oxidative stress and altered bone turnover. Increased osteoclastic activity and decreased osteoblastic activity were shown to be associated with lower level of plasma antioxidants in postmenopausal osteoporosis [37]. It has been experimentally demonstrated that osteoblasts produce antioxidants such as glutathione peroxidase [38]. There has also been evidence that estrogen enhances antioxidant defenses in bone [39]. These findings prove an existing interrelationship between bone turnover and oxidative status in physiological and pathological states, although the exact mechanism remains incompletely understood.

There is evidence that endocrine actions of osteocalcin can be attributed to its undercarboxylated form [5], but unfortunately, we do not have this parameter measured. However, there's also evidence that both carboxylated and uncarboxylated osteocalcin have endocrine effects [40].

In conclusion, we observed the changes in pregnancy that may lead towards atherogenic, prooxidant and insulin resistant state, which are possibly counterbalanced by various protective systems, one of which might be osteocalcin.

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