An Evaluation of Nitric Oxide, Folate, Homocysteine Levels and Lipid Peroxidation in Postmenopausal Osteoporosis

Abstract

Objectives. In the current study, the risk coefficients of nitric oxide (NO), folate, homocysteine levels and lipid peroxidation in postmenopausal osteoporotic women were determined.

Material and Methods. Bone mineral density was determined by dual-energy X-ray absorptiometry. The levels of serum lipid peroxidation products such as the thiobarbituric acid adduct of malondialdehyde (MDA) were measured spectrophotometrically. Plasma folate and homocysteine (Hcy) levels were measured by enzyme chemiluminescence immunoassay. Plasma nitrite levels were measured with the Griess reaction.

Results. The odds ratios and 95% Confidence Intervals (CI) of the variables MDA, folate, NO, body-mass index (BMI), menopause age and age were found significant. MDA, NO and folate variables were found statistically significant in the analysis of receiver operating characteristic (ROCs). The areas under the curve (AUCs, 95% CI) of MDA, NO and folate were found to be statistically significant.

Conclusions. The current study indicates that NO, MDA and folate are risk variables for postmenopausal osteoporosis (Adv Clin Exp Med 2013, 22, 3, 403–409).

Key words: nitric oxide, folate, malondialdehyde, homocysteine, osteoporosis.
Osteoporosis, a major public health problem, is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue [1, 2]. Several risk factors have been identified for osteoporosis such as age, low body mass index (BMI), current smoking, alcohol intake and low bone mineral density (BMD). Deficiencies in folate lead to increased serum concentrations of homocysteine (Hcy), which is associated with bone disorders. Hcy accumulates collagen in bone and contributes to decrease in bone strength. As Tyagi et al. (2011) wrote: „The mechanism of Hcy induced bone loss and remodeling is unclear … Elevated levels of Hcy are associated with various bone abnormalities, such as osteopenia and osteoporosis [3, 4]. The thiol groups in Hcy undergo auto-oxidation, thus triggering oxidative stress and causing the production of reactive oxygen species (ROS). Super oxide anion may react with nitric oxide to produce peroxynitrite with reduced NO bio-availability [5], which may impair the osteoblast–osteoclast balance with unpredictable consequences on bone remodeling” [3]. Folic acid (FA) is a naturally occurring dietary component that reduces the levels of Hcy by increasing the rate of recycling of Hcy to methionine [6]. It is known that Hcy-lowering therapy can favorably influence the course of osteoporosis [7]. In addition, Hcy deficiencies are associated with increased levels of bone turnover markers [8].

During the remodelling cycle, bone is continuously resorbed by osteoclasts and new bone is formed by osteoblasts; this is regulated by local factors such as nitric oxide (NO) [9]. NO is a free radical, synthesized from L-arginine by NO synthases. It has been shown that NO has significant modulatory effects on bone metabolism [10, 11]. It is produced constitutively by osteoblasts [12] and stimulates their proliferation in vitro [13].

Malondialdehyde (MDA), the end product of lipid peroxidation, plays an important role in the process of bone loss and is a measure of osteoclastic activity [14, 15].

Hcy is a thiol-containing amino acid that forms in the process of methionine metabolism. The remethylation pathway that exists for the degradation of Hcy uses folate as a cofactor. Recently, Hcy levels and plasma folate concentrations have been shown to be associated with fracture risk or lower BMD [16, 17].

BMD measurement is widely used for detecting osteoporosis, and dual-energy X-ray absorptiometry (DXA) is a non-invasive method that provides precise BMD assessment [1]. It is likely that BMD is lower in postmenopausal than in pre-menopausal subjects [18].

In the current study, the risk coefficients of nitric oxide, folate, homocysteine levels and lipid peroxidation in postmenopausal osteoporotic women were studied by using the logistic regression method. Receiver Operating Characteristic (ROCs) and areas under the curves (AUCs) also were used to determine the discriminative ability of the variables.

Material and Methods

The Subjects

Postmenopausal women aged 45–65 years (n = 126) who were admitted to the out-patient clinic of gynecology department of faculty of medicine of Dicle University in between April–October 2008 were enrolled in this study. Exclusion criteria were alcohol consumption, tobacco use, postmenopausal hormone replacement therapy, systemic diseases, such as diabetes mellitus and renal failure. All of the subjects had experienced natural menopause; menopause was defined as 1 year with no menstrual bleeding. Routine biochemical analyses (including glucose, urea, creatine, liver function tests, free calcium, and phosphorus) and complete blood count of the subjects were assessed as normal. None of the subjects took any medications likely to affect BMD or calcium metabolism during the study. Baseline characteristics including age, age at menopause, weight, height and body mass index (BMI) were recorded for each subject. A self-administered questionnaire was used to collect information about the subjects’ history as hormone use, smoking status, caffeine consumption, regular alcohol intake, and daily use of milk or milk products. The study was explained to each subject, and written informed consent was read and signed by all the participant, in accordance with the Declaration of Helsinki.

Anthropometric Measurements

The participants’ weight was measured to the nearest 0.1 kg, and their height was measured to the nearest 0.1 cm with a wall-mounted stadiometer (Seca Model 786, Vagel & Halke, Hamburg, Germany) with the subjects wearing light clothing.
and no shoes. Their BMIs (in kg/m²) were then calculated as weight (kg) divided by the square of the height (m²).

**DXA Scanning**

Bone mineral density was determined by dual-energy X-ray absorptiometry (DXA, Discovery QDR 4500A series, Hologic, Waltham, MA, USA, Software 12.3.3 version). Spinal and femoral sites were measured in slow mode and supine position. BMD (g/cm²) was calculated from the bone mineral content (BMC) and bone area (BA). All measurements and analyses were performed by the same experienced investigator. The NHANES III spine and hip reference population for young women (age range 20–40 years) was used. T-Scores were calculated by using a standard formula:

\[ T\text{-score} = \frac{\text{BMD of participant mean} - \text{mean BMD of reference population}}{\text{SD of BMD of reference population}} \]

The diagnostic criteria were derived from World Health Organization criteria for postmenopausal women. Osteoporosis is diagnosed if the bone density at any site has a T-score less than or equal to −2.5. Normal bone density is a T-score greater than −1.0 [19]. The subjects were divided into two groups according to their T-scores: normal and osteoporotic postmenopausal women.

**Blood Samples**

Fasting venous blood samples of the subjects were collected and centrifuged at 3500 rpm for 10 min at room temperature. The samples were stored at −70°C until analysis.

The levels of serum lipid peroxidation products such as the thiobarbituric acid adduct of malondialdehyde (TBA-MDA) were measured spectrophotometrically by a modification of the method described by Buege and Aust [20]. The spectrophotometric measurements were done with Shimadzu UV-1208 spectrophotometer (Japan). The concentrations of lipid peroxidation products were calculated as MDA concentration using the extinction coefficient for the MDA–thiobarbituric acid complex of 1.56×10⁵ l/mol cm at 535 nm.

Plasma tHcy was measured using commercially available kits on an autoanalyzer (Immulite 2500, USA) by enzyme chemiluminescent immunoassay. Plasma folate was analyzed using commercially available kits on an autoanalyzer (Roche E 170, USA), also by enzyme chemiluminescent immunoassay.

Plasma nitrite levels were measured with the Griess reaction [21]. As described in a previous article [22], reduction of nitrate to nitrite was accomplished by catalytic reaction using cadmium. The nitrite produced was determined by diazotization of sulfanilamide and coupling to naphthylethylene diamine. Absorbance of this complex was measured at 545 nm. A standard curve was established with a set of serial dilutions (10⁻⁴–10⁻³ mol/l) of sodium nitrite. A linear regression was performed using the peak area from the nitrite standard. The resulting equation was then used to calculate the sample concentrations. The results were expressed as µmoles per liter plasma.

**Statistical Analysis**

The mean and standard deviation (x ± SD) for continuous variables were calculated. The normality of the variables was analyzed by the Kolmogorov-Smirnov test. The means of variables for the groups of postmenopausal women with and without osteoporosis were tested using Student’s t-test for independent groups.

Binary logistic regression analysis was used to determine the risk factors in osteoporotic postmenopausal women according to total hip T-scores. The subjects’ age, menopause age, folate levels, homocysteine levels, NO levels, BMI and MDA were included when the backward stepwise logistic regression analysis was performed. The variables were considered statistically significant for the model at p < 0.05. ROC curves and areas under the curves were assessed for age, menopause age, folate, NO, BMI and MDA that were found to be significant in the logistic regression model. The ROC curve was applied for folate, NO, and MDA that had high AUCs values. Odds ratios were calculated by the logistic regression method. Two-sided p-values were considered statistically significant at p < 0.05. The statistical analyses were carried out using the statistical packages for SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL, USA).

**Results**

There were significant differences between the two groups, in age, menopause age, BMI, MDA, folate, NO (P < 0.05). The osteoporotic markers were presented in Table 1. Differences in femur BMD (FBMD), vertebra BMD (VBMD), vertebra BMC (VBMC), femur BMC (FBMC) and vertebra T-score (VT-score) were also found to be statistically significant (p < 0.001).

Table 2 shows the risk ratios extracted by using logistic regression method in postmenopausal women with or without osteoporosis. The results showed that MDA, folate, NO, BMI, menopause age and age are significant risks in postmenopausal
women with osteoporosis. The odds ratios and 95% confidence intervals (CI) of those variables (MDA, folate, NO, BMI, menopause age and age) were found to be significant and were as follows: 1.682 (1.06 – 2.66), 1.373 (1.01 – 1.87), 1.575 (1.29 – 1.93), 1.925 (1.57 – 2.35), 1.526 (1.28 – 1.82) and 1.261 (1.07 – 1.49), respectively. The ratio of correct classification was 0.9675 (Table 2).

The variables found significant in the analysis of logistic regression were then analysed using ROCs. The ROC curves are presented in Figure 1. MDA, NO and folate were the variables that were found to be significant in the analysis of ROCs. The AUCs (95%CI) values of MDA, NO and folate were found to be significant, as follows: 0.782 (0.657 – 0.907), 0.713 (0.568 – 0.857) and 0.744 (0.608 – 0.880), respectively.

**Discussion**

In the current study, the variables which constituted risk for osteoporosis in post menopausal women were determined by using logistic regression and receiver operating characteristic (ROCs) with the areas under the curves (AUCs). The statistical analyses for NO, folate and MDA revealed statistically significant results for postmenopausal osteoporotic women.

As Armour et al. wrote: “Nitric oxide (NO) is a pleiotropic signaling molecule that has potent effects on osteoblast and osteoclast activity in vitro” [23]. Previous studies conducted on both animal and human models have shown that NO is an important regulator on bone cell function [9, 11, 24]. Hao et al. noted that “NO has biphasic effects on osteoclastic bone resorption, and low concentrations of NO augment interleukin-1-induced
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However, as Van’t Hoff et al. pointed out, “High concentrations of NO inhibit osteoclast formation and activity” [11].

Increasing levels of MDA (in plasma and erythrocyte) have been shown to be a result of lipid peroxidation in osteoporotic females compared with a non-porotic control group [14, 15]. Sontakke and Tare [26] found elevated levels of MDA in a group of postmenopausal osteoporotic individuals compared to healthy controls, indicating the potential role of lipid peroxidation in bone metabolism. However, Maggio et al. [27] has reported similar MDA levels in aged osteoporotic women compared to non-porotic controls.

In highly stressed conditions like increased osteoclastic activity, NO can react with oxygen-derived free radicals such as superoxide anions to form highly reactive molecules. The production of such toxic moieties may contribute to the tissue damage by inducing lipid peroxidation [14, 15]. This may explain the increased NO and MDA levels in osteoporotic women.

Ravaglia et al. [28] found that low serum folate is responsible for the risk of osteoporotic fracture, indi-

<table>
<thead>
<tr>
<th>Variables (Zmienne)</th>
<th>β</th>
<th>SE</th>
<th>Wald</th>
<th>OR (95% CI) p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>0.522</td>
<td>0.234</td>
<td>4.97</td>
<td>1.682 (1.06–2.66) 0.027</td>
</tr>
<tr>
<td>Folate (Kwas foliowy)</td>
<td>0.322</td>
<td>0.156</td>
<td>4.26</td>
<td>1.373 (1.01–1.87) 0.021</td>
</tr>
<tr>
<td>NO (Tlenek azotu)</td>
<td>0.457</td>
<td>0.101</td>
<td>20.47</td>
<td>1.575 (1.29–1.93) &lt; 0.001</td>
</tr>
<tr>
<td>BMI</td>
<td>0.655</td>
<td>0.102</td>
<td>41.24</td>
<td>1.925 (1.57–2.35) &lt; 0.001</td>
</tr>
<tr>
<td>Menopause age (Wiek menopauzy)</td>
<td>0.423</td>
<td>0.093</td>
<td>22.09</td>
<td>1.526 (1.28–1.82) &lt; 0.001</td>
</tr>
<tr>
<td>Age (Wiek)</td>
<td>0.232</td>
<td>0.085</td>
<td>7.449</td>
<td>1.261 (1.07–1.49) &lt; 0.001</td>
</tr>
</tbody>
</table>

Correct classification (Poprawna klasyfikacja): 96.75%

Fig. 1. ROC curves, AUC values and standard error, p value and 95% CI of AUC of MDA, NO and folate

Ryc. 1. Krzywe ROC, wartości AUC i błąd standardowy, wartość p oraz 95% CI AUC dla MDA, NO i kwasu foliowego
cating the role of folate in BMD. Cagnacci et al [29] observed a significant relation between folate and BMD in the lumbar spine, as plasma folate levels were decreased in the osteoporotic group as compared with the non osteoporotic controls. This coincides with a study of 271 postmenopausal Iranian women by Golbahar et al., in which BMD exhibited a positive correlation with serum folate [30].

In the current study, the risk ratios of NO, MDA and folate in postmenopausal women with osteoporosis were found to be significant (1.575, 1.682 and 1.373, respectively). These risk ratios show the real amount of NO, MDA and folate in osteoporotic women. The present study has established that these important risk variables have the discriminative ability to identify women with or without osteoporosis. In fact, the discriminative ability (AUCs values) of MDA, NO, and folate were found to be 0.78, 0.71 and 0.74, respectively, in the results of the ROCs. These distinct outcomes show the risk of osteoporosis in postmenopausal women in detail.

Recent data suggest that homocysteine (Hcy) and folate affect bone metabolism in humans [17]. Yilmaz et al. showed that serum Hcy levels were significantly higher in postmenopausal osteoporotic women than their non-osteoporotic counterparts [31]. Similarly, the increased risk of osteoporosis in case of high homocysteine blood levels was emphasized by Leboff et al. [32]. However, several investigations reported no associations, or only weak or inversed associations between Hcy and bone mineral density (BMD) [29, 30, 33]; the current study showed no relation between Hcy and BMD. In the current study the mean age of postmenopausal women was 62.9 ± 6.6 years, whereas in the study of Van Meurs et al. [34], the subjects were over 70 years of age. As Herrmann et al. pointed out: „However, [the lack of interaction between Hcy and BMD] is not surprising, since BMD mainly reflects bone mineralization and provides only an integral measure of bone metabolism” [35].

In addition, the current study found that age, menopause age and BMI were also significant predictors of BMD in postmenopausal women. The negative association of BMD with age and positive association with BMI have already been documented [36, 37].

The authors concluded that NO, MDA and folate are risk variables and the detailed risk ratios of these factors on the postmenopausal osteoporotic women have also been presented.

References


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