

**NITRIC OXIDE PRODUCTS ARE NOT ASSOCIATED
WITH METABOLIC SYNDROME**

PRODUKTI AZOT-MONOKSIDA NISU POVEZANI SA METABOLIČKIM SINDROMOM

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– Faculty of Medicine, Belgrade, Serbia⁵Department of Medical Biochemistry, University of Belgrade – Faculty of Pharmacy, Belgrade, Serbia**Summary**

Background: Nitric oxide (NO) is oxidative stress biomarker which is regarded as one of the key determinants of energy metabolism and vascular tone. Considering the controversial reports on the association between nitric oxide products (NOx) and metabolic syndrome (MetS), the aim of the current study was to examine that potential relationship. Additionally, we aimed to evaluate a broad spectrum of other oxidative stress biomarkers [i.e., malondialdehyde (MDA), advanced oxidation protein products (AOPP), xanthine oxidoreductase (XOD), xanthine oxidase (XO) xanthine dehydrogenase (XDH)] in relation with MetS.

Methods: A total of 109 volunteers (46.8% of them with MetS) were included in this cross-sectional study. Biochemical and anthropometric parameters, as well as blood pressure, were obtained. The MetS was diagnosed according to the International Diabetes Federation criteria.

Results: Multivariate logistic regression analysis showed that XOD (OR=1.011; 95% CI 1.002–1.019; p=0.016), XO (OR=1.014; 95% CI 1.003–1.026; p=0.016), MDA (OR=1.113; 95% CI 1.038–1.192; p=0.003) and AOPP (OR=1.022; 95% CI 1.005–1.039; p=0.012) were the independent predictors of MetS, whereas no association between NOx and MetS was found. As XOD rose for 1 U/L, XO for 1 U/L, MDA for 1 μmol/L and AOPP for 1 T/L, probability for MetS rose for 1.1%, 1.4%, 11.3% and

Kratak sadržaj

Uvod: Azot-monoksid (NO) je biomarker oksidativnog stresa i smatra se jednom od ključnih determinanti energetskeg metabolizma i vaskularnog tonusa. S obzirom na oprečne rezultate dosadašnjih studija koje se odnose na povezanost između produkata azot-monoksida (NOx) i metaboličkog sindroma (MetS), cilj ove studije je da se ispita njihova potencijalna povezanost. Takođe, cilj je i da se ispita povezanost između širokog spektra ostalih biomarkera oksidativnog stresa [npr., malondialdehida (MDA), produkata uznapredovale oksidacije proteina (AOPP), ksantin oksidoreduktaze (XOD), ksantin oksidaze (XO) i ksantin dehidrogenaze (XDH)] i MetS.

Metode: Ukupno 109 dobrovoljaca (od kojih je 46,8% ispunjavalo kriterijume za MetS) je uključeno u studiju preseka. Mereni su biohemijski i antropometrijski parametri, kao i krvni pritisak. Dijagnoza MetS-a je postavljena prema kriterijumima Međunarodnog udruženja za dijabetes.

Rezultati: Multivarijantna logistička regresija je pokazala da su XOD (OR=1,011; 95% CI 1,002–1,019; p=0,016), XO (OR=1,014; 95% CI 1,003–1,026; p=0,016), MDA (OR=1,113; 95% CI 1,038–1,192; p=0,003) i AOPP (OR=1,022; 95% CI 1,005–1,039; p=0,012) nezavisni prediktori MetS-a, ali postojanje povezanosti između NOx i MetS nije utvrđeno. Sa porastom XOD za 1 U/L, XO za 1 U/L, MDA za 1 μmol/L i AOPP za 1 T/L verovatnoća

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2.2%, respectively. Adjusted R^2 for the Model was 0.531, which means that 53.1% of variation in MetS could be explained with this Model.

Conclusions: Unlike XOD, MDA and AOPP, NOx is not associated with MetS.

Keywords: inflammation, metabolic syndrome, obesity, oxidative stress

Introduction

Metabolic syndrome (MetS) has reached an alarming rate, parallel with increasing obesity and type 2 diabetes (DM2), thus representing one of the major public health concerns (1).

Considering the fact that abdominal obesity is the key characteristic of MetS (2), it is assumed that visceral adipose tissue through increased mitochondrial oxidation of free fatty acids represents the main generator of reactive oxygen (ROS) and reactive nitrogen species (RNS) (3). Indeed, previous studies confirmed an increase in some of the oxidative stress biomarkers, as well as a decrease in the antioxidant defence system in individuals with overweight/obesity (4, 5). However, it is questionable whether all of these biomarkers can predict MetS since discrepancies in the literature exist considering the relationship between oxidative stress biomarkers and MetS and/or its components (6–16).

Nitric oxide (NO) is an oxidative stress biomarker which is regarded as one of the key determinants of energy metabolism and vascular tone (12). It is generated from L-arginine, and this production is under the control of enzyme NO synthase (NOS) (12). NOS exists in three different isoforms (i.e., inducible, endothelial and neuronal) which all may have an impact on the bioavailability of NO in circulation (6, 12). However, it is believed that inducible NOS has the highest capacity for NO generation whose expression is increased in response to inflammation and oxidative stress (12).

Some studies report the decrease in serum NO (11, 12), while others showed its increase in metabolic disorders (7, 9). In addition, the relationship between serum nitric oxide products (NOx) and body fat, rather than between serum NOx and lipid parameters was found (8).

Taking into account controversial results on the association between NOx (nitrates and nitrites) and MetS, the aim of the current study was to examine that potential relationship. Additionally, we aimed to evaluate a broad spectrum of other oxidative stress biomarkers [i.e., malondialdehyde (MDA), advanced oxidation protein products (AOPP), xanthine oxidoreductase (XOD), xanthine oxidase (XO) xanthine dehydrogenase (XDH)] in relation with MetS.

pojave MetS-a je rasla za 1,1%, 1,4%, 11,3% i 2,2%. Prilagođeni R^2 za Model iznosio je 0,531, što ukazuje na to da čak 53,1% varijabilneta u MetS-u može biti objašnjeno ovim modelom.

Zaključak: Za razliku od XOD, MDA i AOPP, NOx nisu povezani sa MetS-om.

Ključne reči: inflamacija, metabolički sindrom, gojaznost, oksidativni stres

Materials and Methods

Study population

A total of 109 volunteers (62.3% of them females) were recruited in the research when visiting the Primary Health Care Center in Podgorica, Montenegro, for their routine check-up in the period from October 2015 to May 2016. Clinical examinations and medical history were carried out on the same day for each participant.

The diagnosis of MetS was established according to the International Diabetes Federation criteria (17). The examinees that were eligible to enter the study were divided into two groups: a group without MetS (n=58) and a group with MetS (n=51).

Exclusion criteria for all potential participants were: hsCRP >10 mg/L, acute inflammatory disease, diabetes mellitus, liver diseases other than steatosis, kidney diseases, malignant diseases, gout, ethanol consumption >20 g/day, pregnancy, as well as unwillingness to participate in the study.

The Ethical Committee of the Primary Health Care Center in Podgorica, Montenegro approved the study protocol. All the volunteers provided signed informed consent, and the research was carried out in compliance with the Declaration of Helsinki.

Anthropometric and blood pressure measurements

Basic anthropometric measurements, as well as systolic (SBP) and diastolic blood pressure (DBP) were obtained as described elsewhere (18).

Biochemical analyses

Biochemical analyses were performed after overnight fasting of at least 8 hours. A cubital venous blood sample was collected from each participant, as previously described (18).

Lipid parameters [e.g., total cholesterol (TC), high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), triglycerides (TG)], glucose, creatinine, uric acid, bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT), were measured using standardized enzymatic proce-

dure (Roche Cobas 400, Mannheim, Germany). HsCRP levels were measured nephelometrically (Behring Nephelometer Analyzer, BN II, Marburg, Germany).

Serum MDA level and catalase (CAT), XOD, and XO activity were measured by spectrophotometric assay. Determination of MDA was based on measuring TBARS by thiobarbituric acid (TBA) test (19). Determination of XOD and XO in serum was based on the liberation of uric acid by using xanthine as a substrate in the presence of NADH (for XOD) or absence of NADH (for XO) when only molecular oxygen was electron acceptor (20). The XDH activity was calculated by subtracting XO from XOD activity, and the results in XOD, XO and XDH, respectively were expressed in U/L. Determination of serum AOPP was based on spectrophotometric detection of chloramine-T equivalents. In order to minimise the impact of storage time of samples, as well as the possible influence of TG and turbidity of samples, we modified AOPP assay by precipitating VLDL and LDL in the plasma (21).

Serum NO_x was determined as follows: nitric oxide is rapidly converted to nitrite (NO₂⁻) and nitrate (NO₃⁻) in human serum. The measurement of these anions, commonly named as NO_x, is used as an indicator of NO production. In our study, serum NO_x levels were measured as previously described (22) after chemical reduction of nitrate to nitrite by Cu-coated cadmium, followed by a colourimetric detection at 540 nm of nitrite as the azo dye product of the Griess reaction. The detection limit of the assay was 2.5 μmol/L.

Catalase (CAT) test was based on the release of oxygen from hydrogen peroxide (H₂O₂), by using the spectrophotometric assay based on the formation of its stable complex with ammonium molybdate (23).

Statistical analysis

Statistical analysis was performed with SPSS 22 Statistical Package Program for Windows (SPSS Inc, Chicago, Illinois). The parametric Student *t*-test and non-parametric Mann-Whitney *U* test were used to test differences between two groups dependent on the variables distributions. Kolmogorov-Smirnov test was used for distribution testing. Data are shown as mean ± standard deviation for normally distributed continuous variables, as geometrical mean (95% confidence interval) for log-normally distributed variables (24), and median (interquartile range) for skewed distributed data. The comparisons of absolute frequencies were performed using Chi-square test for contingency tables. Logistic regression analysis was used to determine possible associations between MetS and oxidative stress and inflammation parameters. Continuous variables which were significantly different between groups and had not entered the algorithm for MetS diagnosis were classified as confounders and applied in multivariate logistic regression models in order to identify independent predictors of MetS occurrence. The Hosmer and Lemeshow test was used to examine whether there was a linear relationship between the confounders and the log odds of the dependent variable. Results are given as odds ratio (OR) with 95% confidence intervals (CI). The explained variation in MetS occurrence was given by Nagelkerke *R*² value. *P* < 0.05 (two-tailed) was considered statistically significant.

Results

A summary of general demographic characteristics is listed in *Table 1*. Significantly higher BMI and WC (*p*<0.001, for both), SBP (*p*<0.001) and DBP

Table 1 Basic demographic characteristics of participants without and with metabolic syndrome.

	Participants without MetS	Participants with MetS	<i>p</i>
N (male/female)	58 (20/38)	51 (21/30)	0.602
Age, years*	55 (40–68)	60 (53–66)	0.266
BMI, kg/m ² **	26.21 (24.99–27.50)	30.47 (29.40–31.59)	<0.001
WC, cm	94.78±13.42	108.45±13.84	<0.001
SBP, mmHg*	130 (136–136)	141 (135–148)	<0.001
DBP, mmHg*	77 (70–80)	80 (72–90)	0.010
Antihypertensives (No/Yes)	28/21	30/30	0.582
Hypolipidemic drugs (No/Yes)	48/10	33/18	0.053
Smoking habits, (Smoker/Non-smoker)	46/12	36/15	0.406

Data are presented as arithmetic mean ± SD and compared by Student *t*-test.

* Skewed distributed data are presented as median (interquartile range) and compared by Mann-Whitney test.

** Log – normal distributed data are presented as geometric mean (95% CI) compared by Student *t*-test. Antihypertensives, hypolipidemic drugs and smoking habits are given as absolute frequencies and compared by Chi-square test for contingency tables.

BMI – Body mass index; WC – Waist circumference; SBP – Systolic blood pressure; DBP – Diastolic blood pressure

Table II Clinical characteristics of participants without and with metabolic syndrome.

	Participants without MetS	Participants with MetS	p
Glucose, mmol/L*	5.20 (4.90–5.80)	5.90 (5.40–6.47)	<0.001
HbA1c, %**	4.94 (4.80–5.08)	5.40 (5.29–5.55)	<0.001
TC, mmol/L	5.52±1.14	5.74±1.33	0.345
HDL-c, mmol/L	1.59±0.42	1.34±0.36	<0.001
LDL-c, mmol/L**	3.18 (2.95–3.43)	3.39 (3.07–3.74)	0.303
TG, mmol/L**	1.29 (1.13–1.46)	1.91 (1.73–2.10)	<0.001
hsCRP, mg/L*	1.24 (0.97–1.58)	2.19 (1.77–2.72)	0.001
Bilirubin, µmol/L**	7.46 (6.62–8.41)	7.16 (6.31–8.14)	0.644
Uric acid, µmol/L	264.07±74.77	316.94±68.34	<0.001
Creatinine, µmol/L**	69.31 (65.57–73.27)	73.27 (69.10–77.70)	0.171
eGFR _{MDRD} , mL/min/1.73 m ²	88.22±20.90	82.69±18.44	0.148
AST, U/L*	19 (17–22)	21 (18–26)	0.044
ALT, U/L*	18 (14–23)	25 (19–35)	<0.001
GGT, U/L*	13 (12–18)	19 (14–30)	0.001
XOD, U/L	325.34±61.79	351.60±72.77	0.044
XO, U/L*	127.40 (110.25–142.10)	144.55 (122.50–175.15)	0.012
XDH, U/L	194.48±80.05	199.94±83.10	0.728
MDA, µmol/L	49.74±7.28	54.15±8.25	0.004
AOPP, T/L	67.81 (58.23–118.17)	74.70 (68.17–130.53)	0.010
CAT, U/L	75.06±45.19	66.92±43.54	0.342
NOx, µmol/L	37.69 (32.47–54.69)	40.02 (33.36–49.69)	0.913

Data are presented as arithmetic mean ± SD and compared by Student t-test.

* Skewed distributed data are presented as median (interquartile range) and compared by Mann-Whitney test.

** Log-normal distributed data are presented as geometric mean (95% CI) compared by Student t-test.

HbA1c-Glycated hemoglobin; TC-Total cholesterol; HDL-c-High density lipoprotein cholesterol; LDL-c-Low density lipoprotein cholesterol; TG-Triglycerides; hsCRP-High-sensitivity C-reactive protein; eGFR_{MDRD}-Estimated glomerular filtration rate; AST-Aspartate aminotransferase; ALT-Alanine aminotransferase; GGT-Gamma-glutamyl transferase; XOD-Xanthine oxidoreductase; XO-Xanthine oxidase; XDH-Xanthine dehydrogenase; MDA- Malondialdehyde; AOPP-Advanced oxidation protein products; CAT-Catalase; NOx-Nitric oxide products

($p=0.010$) were evident in the group of participants with MetS. There was no significant difference between the age of individuals in different groups. No significant unequal distributions of gender, participants with smoking habits and therapy usages were evident between tested groups.

Fasting glucose ($p<0.001$), HbA1c ($p<0.001$), TG ($p<0.001$), uric acid concentrations ($p<0.001$), AST, ALT and GGT activities ($p=0.044$, $p<0.001$ and $p=0.001$, respectively) were significantly higher in participants with MetS when compared with participants without MetS. Also, markers of oxidative stress such as XO, XOD, MDA and AOPP were evidently higher in participants with MetS ($p=0.044$, $p=0.012$, $p=0.004$ and $p=0.010$, respectively). There was no difference in NOx levels between participants with and without MetS ($p=0.913$). Opposite to this, par-

ticipants with MetS had a lower HDL-c concentration ($p<0.001$) (Table II).

Table III presented results of logistic regression analysis which was performed in order to examine the associations of oxidative stress and inflammation parameters (predictors) and MetS (without MetS coded 0, with MetS coded 1) as a dependent variable. In the unadjusted model, predictors significantly associated with MetS occurrence were hsCRP (OR=1.306; 95% CI 1.052–1.621; $p=0.016$), XOD (OR=1.006; 95% CI 1.000–1.012; $p=0.047$), XO (OR=1.010; 95% CI 1.001–1.020; $p=0.025$) and MDA (OR=1.078; 95% CI 1.022–1.137; $p=0.006$). As hsCRP rose for 1 mg/L, XOD for 1 U/L, XO for 1 U/L and MDA for 1 µmol/L probability for MetS rose for 30.6%, 0.6%, 1.0% and 7.8%, respectively. No significant associations were determined between

Table III Odds ratios (OR) after univariate and multivariate logistic regression analysis for clinical parameters predicting metabolic syndrome.

Predictors	Unadjusted OR (95% CI)	P	Nagelkerke R ²
hsCRP, mg/L	1.306 (1.052–1.621)	0.016	0.080
XOD, U/L	1.006 (1.000–1.012)	0.047	0.050
XDH, U/L	1.001 (0.996–1.006)	0.725	0.002
XO, U/L	1.010 (1.001–1.020)	0.025	0.068
MDA, mmol/L	1.078 (1.022–1.137)	0.006	0.102
AOPP, T/L	1.009 (0.998–1.020)	0.095	0.034
CAT, U/L	0.996 (0.987–1.004)	0.340	0.011
NOx, mmol/L	1.002 (0.985–1.019)	0.843	0
Model	Adjusted OR (95% CI)	P	Nagelkerke R ²
hsCRP, mg/L	1.199 (0.922–1.560)	0.175	0.531 (for model)
XOD, U/L	1.011 (1.002–1.019)	0.016	
XO, U/L	1.014 (1.003–1.026)	0.016	
MDA, mmol/L	1.113 (1.038–1.192)	0.003	
AOPP, T/L	1.022 (1.005–1.039)	0.012	

Model: HsCRP, uric acid, ALT, GGT, XOD, XO, MDA and AOPP

hsCRP – High-sensitivity C-reactive protein; XOD – Xanthine oxidoreductase; XO – Xanthine oxidase; XDH – Xanthine dehydrogenase; MDA – Malondialdehyde; AOPP – Advanced oxidation protein products; CAT – Catalase; NOx – Nitric oxide products

XDH, AOPP, CAT, NOx and MetS (Table III). Multivariate logistic regression analysis was performed in order to test if predictors which were significant in univariate regression analysis confounded with other clinical parameters which could be independently associated with MetS occurrence. AST was not included in the logistic regression model because the Hosmer-Lemeshow test was significant ($p < 0.05$) for the Model indicating that there was no linear relation-

ship between the confounders and the *log* odds of the dependent variable. Also, BMI, WC, SBP, DBP, lipid profile parameters, fasting glucose and HbA1c were excluded from the logistic regression analysis because they were used in MetS diagnosis algorithm. Multivariate logistic regression analysis showed that XOD (OR=1.011; 95% CI 1.002–1.019; $p=0.016$), XO (OR=1.014; 95% CI 1.003–1.026; $p=0.016$), MDA (OR=1.113; 95% CI 1.038–1.192; $p=0.003$) and AOPP (OR=1.022; 95% CI 1.005–1.039; $p=0.012$) were the independent predictors of MetS. As XOD rose for 1 U/L, XO for 1 U/L, MDA for 1 μ mol/L and AOPP for 1 T/L, probability for MetS rose for 1.1%, 1.4%, 11.3% and 2.2%, respectively. HsCRP lost its independent prediction on MetS occurrence (OR=1.199; 95% CI 0.922–1.560; $p=0.175$) in multivariate logistic regression analysis. Adjusted R² for the Model was 0.531, which means that 53.1% of the variation in MetS could be explained with this Model (Table III). Observed post-hoc power of the study based on multiple regression analysis for 8 predictors, probability level 0.05 and R² = 0.531 was 0.99 (25).

Discussion

The findings of the current study showed an independent association of different oxidative stress biomarkers with MetS (Table III). However, NOx showed no association with MetS.

In our study, we have confirmed that both, MDA and AOPP were reliable predictors of MetS in non-diabetic participants, showing that proteins are equally targeted by ROS as lipids. Furthermore, we have also shown the increase in XOD and XO in individuals with MetS, and both of these biomarkers were shown to be the independent predictors of MetS (Table III).

Previous studies that examined the impact of different oxidative stress biomarkers on MetS status lack consistency (6–16). Venturini et al. (13) did not find the increase in MDA (i.e., a primary biomarker of lipid peroxidation) in individuals with obesity, but did in MetS, thus suggesting that a cluster of cardio-metabolic risks, rather than obesity *per se*, could lead to the apparent increase in MDA level. On the other hand, Abdilla et al. (14) found no relationship between MDA and each MetS component, but only a weak association with BMI.

Regarding AOPP as a marker of oxidative damage of proteins, previous studies were consistent and showed its increase in MetS (15, 26). Moreover, Venturini et al. (26) reported the superiority of AOPP over lipid peroxidation biomarkers in relation to MetS components.

To our knowledge, only one study examined the XO activity in relation with MetS, so far. Although the latter study included a smaller sample size of examinees than ours, we have confirmed their results, thus

showing an increase in XO activity in MetS (27). This enzyme can contribute to the controlling of vascular tone and endothelial function by modulating the bioavailability of endothelial NO. Moreover, XO is involved in adipogenesis, as well as in the production of uric acid, which all can explain the role of XO in the pathogenesis of cardiometabolic diseases (28).

However, the most contradictory results were shown regarding NOx. While one group of authors reported the decrease of NOx in the state of obesity and diabetes (11, 12), the other claimed the opposite, showing its increase (7, 9) in individuals with MetS and diabetes. Our results are similar to the third group of researchers who found a non-significant increase of NOx production in obesity, diabetes and MetS (29, 30). Namely, Caimi et al. (30) found no difference between diabetic and non-diabetic individuals with MetS. On the other hand, Ueyama et al. (6) found the inverse correlation between NOx and HDL-c only in males, but not in females. However, no relationship between serum NOx levels and other MetS components, such as high BMI, high TG, or hypertension was reported (6). Furthermore, Chedraui et al. (7) also found the inverse correlation between NOx and HDL-c, but a strong positive correlation with TG in the cohort of examined postmenopausal women.

Different populations of studied groups and their different sample-size, may at least in part explain these discrepancies. Also, NOS, the enzyme responsible for NO production, exists in three isoforms, but it is suggested that inducible NOS, rather than endothelial or neuronal NOS, is the main isoform whose expression is increased in oxidant and inflam-

matory states, such as obesity (31). However, the high sensitivity of NO to ROS results in generating other reactive species, which may also influence the bioavailability of NOx (31). In addition, a problem concerning distinguishing between NO production from constitutive or inducible NOS isoforms, may also partly explain discrepancies between studies.

The cross-sectional design is the limitation of our study which does not allow us to establish the causal link between oxidative stress and MetS. However, our study has some advantages, since we have included a broad spectrum of oxidative stress biomarkers in relation with MetS. In addition, only one study, but with a smaller sample size than ours, examined the role of XO in MetS. Thus, new studies with longitudinal design are needed to confirm our results.

Conclusion

The finding of the current study revealed an independent relationship of different oxidative stress biomarkers with MetS occurrence. However, no association between NOx and MetS was found.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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