3-nitrotyrosine as a serum biomarker of nitroxidative stress and insulin resistance in nascent metabolic syndrome

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Metabolic syndrome (MetS) represents a cluster of metabolic abnormalities, including central obesity, hypertension, hypertriglyceridemia, low HDL cholesterol and hyperglycaemia. There is increasing evidence that nitroxidative stress is closely related to MetS and its metabolic and cardiovascular complications. 3-nitrotyrosine (NT), a stable product of a posttranslational protein modification, has appeared as a marker of nitroxidative stress. Studies on circulating NT levels in MetS are limited and discordant. Therefore, the aim of the current study was to determine the serum NT levels in patients with nascent MetS. It also revealed the association between NT, serum fasting glucose and homeostasis model assessment (HOMA-IR)-estimated insulin resistance.

The study involved 63 patients with nascent MetS and 34 healthy controls. Serum NT concentrations were determined using an ELISA method. The levels of NT did not differ significantly between patients with MetS and the control group [12.59 (3.79–22.68) nmol/L vs. 4.23 (1.73–18.32) nmol/L; \( p = 0.08 \), respectively]. However, subjects with five MetS components had significantly higher NT concentrations [28.13 (19.27–38.58) nmol/L; \( n = 20 \)], compared to patients with only three MetS components [6.24 (1.8–9.8) nmol/L; \( n = 17 \), \( p < 0.0001 \)] and to controls (\( p < 0.0001 \)).

A significant positive correlation between NT concentrations and glycaemia was evident only in the presence of all five MetS components (\( r = 0.485, p = 0.03 \)). Nitrotyrosine correlated positively with HOMA-IR in all MetS patients (\( r = 0.287, p = 0.025 \)).

Nitroxidative stress might be associated with insulin resistance in subjects with MetS and increases proportionally to the number of components of the syndrome.

Keywords: nitrotyrosine, nitroxidative stress, metabolic syndrome, insulin resistance.

INTRODUCTION

Metabolic syndrome (MetS) represents a constellation of metabolic disturbances, comprising central obesity, hypertension, dyslipidemia – hypertriglyceridemia and low levels of high-density lipoprotein (HDL) cholesterol, and hyperglycaemia [1]. Although there has been a significant debate regarding the criteria and concept of the syndrome, it is unequivocally linked to an increased risk of developing type 2 diabetes (T2DM) and cardiovascular diseases (CVD). It is generally accepted that insulin resistance (IR) is the major underlying mechanism responsible for development of MetS [2, 3]. IR is the pivotal predisposing factor and the best indicator of future onset of T2DM, since it normally precedes impaired glucose tolerance (IGT) and fasting hyperglycaemia [3]. The precise molecular mechanisms that link metabolic abnormalities observed in MetS to IR and CVD have not been clarified yet. Many lines of evidence suggest that oxidative stress (OxS) may represent such a link, since it is implicated in each individual component of the syndrome [3, 4]. Increased OxS appears to be a deleterious factor resulting in decreased peripheral insulin sensitiv-
It is also well established that inflammation is closely related to OxS, since the pathways, generating mediators of inflammation such as interleukins (IL), cytokines and adhesion molecules, are induced by OxS. Moreover, chronic low-grade inflammation has been recognized as another common event in the unifying pathogenic view for MetS [4].

Given the dramatic increase in the prevalence of MetS worldwide, the evaluation of oxidant-mediated biomolecule modifications which can also predict clinical outcomes is beneficial. However, the classical definition of OxS as a loss of balance between ROS formation and antioxidant defense [9], does not emphasize on the pivotal role of nitric oxide (•NO) and reactive nitrogen species (RNS) in the pro-oxidative alterations. A relevant oxidative posttranslational protein modification, initialized by •NO, is the nitration of protein tyrosine (Tyr) residues to 3-nitrotyrosine (NT), performed through peroxynitrite and myeloperoxidase (MPO)-dependent nitration pathways [10]. A new term – “nitroxidative stress” has been introduced to reinforce the concept that nitration is caused by •NO-derived oxidants and will be used herein [11]. The nitroxidative stress may represent another mechanism involved in IR and MetS pathogenesis and may also indirectly link the syndrome to its later complications such as T2DM, atherosclerosis and CVD. All of the above mentioned pathologies as well as IR and hyperglycaemia, are associated with increased yield of ROS and RNS, endothelial dysfunction, pro-inflammatory changes and decreased bioavailability of •NO [11, 12]. Furthermore, elevated plasma NT levels have also been documented in diabetes [13] and in high-fat diet [14]. Moreover, the hypothesis for involvement of nitroxidative stress in CVD, has also been supported by an observed strong correlation between circulating protein NT, atherosclerotic risk and prevalence of coronary artery disease (CAD) [15].

The provided data suggest involvement of nitroxidative stress in the pathogenesis of MetS and also highlight the potential of NT to monitor progression of MetS to MetS-related complications. However, studies on serum NT levels in MetS are limited and discordant. It is also unclear whether the accumulation of factors related to MetS increases the degree of underlying nitroxidative stress. Therefore, the aim of the current study was to determine the serum levels of NT in patients with nascent MetS. We also postulated a correlation between NT, serum fasting glucose and homeostasis model assessment (HOMA-IR)-estimated insulin sensitivity.

**EXPERIMENTAL**

**Subjects:** The study involved 63 patients with nascent MetS, uncomplicated with T2DM, admitted to the Department of Endocrinology and Metabolic Disorders, University Hospital “Kaspela”, Plovdiv, Bulgaria. Patients were diagnosed as having MetS according to the International Diabetes Federation (IDF) global consensus definition, modified by the joint statement of the National Heart, Lung and Blood Institute; the American Heart Association; the World Heart Federation; the International Atherosclerosis Society and the International Association for the Study of Obesity. The presence of any three or more of the following five parameters were considered as diagnostic of MetS: (1) waist circumference: ≥ 94 cm in men, ≥ 80 cm in women; (2) elevated triglyceride (TG) levels ≥1.7 mmol/L or drug treatment for elevated TG; (3) reduced HDL cholesterol: <1.0 mmol/L in men, <1.3 mmol/L in women or history of specific treatment for this lipid abnormality; (4) elevated blood pressure (BP): systolic BP ≥130 mm Hg or diastolic BP ≥85 mmHg or drug treatment for hypertension; and (5) fasting blood glucose (FG) >5.6 mmol/L or drug treatment for hyperglycaemia [1]. The control group consisted of 34 healthy sex- and age-matched subjects who had no history of heart disease, diabetes, hypertension, obesity and smoking. The control subjects were not taking any medications or antioxidant supplements. All the enrolled in the study participants met the following inclusion criteria: have no acute or chronic infections or inflammatory disease, no active immunological disease, no advanced CVD, no other known chronic illness, pregnancy or alcohol abuse.

The study was approved by the Human Ethics Committee of Medical University – Plovdiv (№4/21.09.2017) and was conducted in accordance with the Declaration of Helsinki. All participants signed an informed consent.

**Laboratory analysis:** Fasting blood samples in anticoagulant-free tubes and clinical data were collected. Thirty minutes after blood collection tubes were centrifuged at 3000 g for 10 minutes and se-
rum was separated in Eppendorf tubes. Serum samples for NT detection were stored at −80 °C until analysis. Serum FG concentrations were measured immediately by the standard hexokinase enzymatic method. Serum FG, TG, total and HDL cholesterol were analyzed using an automatic blood analyzer (Beckman Coulter AU480, Beckman Instruments Inc., USA). Serum insulin levels were determined by chemiluminescent immunoassay (Beckman Coulter Access, Beckman Instruments Inc., USA). The homeostasis model assessment (HOMA-IR) was used to detect the degree of IR by measuring the levels of FG and insulin. HOMA-IR was calculated using the following formula: HOMA-IR = (fasting glucose [mmol/L]×fasting insulin [μU/mL]) / 22.5 [16].

The concentrations of serum protein-bound NT (nmol/L) were measured by ELISA method using commercially available kit (Hycult Biotech, Uden, the Netherlands), according to the manufacturer’s instructions. The absorbance was red at 450 nm on ELISA reader (HumaReader HS, HUMAN, Wiesbaden, Germany). Concentrations of unknown samples were determined using a standard curve, constructed by plotting absorbance values versus concentrations of standards.

Statistics: Statistical analysis was performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to evaluate whether the distribution of continuous variables was normal. Student’s t-test and the Mann-Whitney U test were used for the analysis of parametric and non-parametric data, respectively. Continuous variables were expressed as mean ± SD or as median and interquartile range. Spearman’s rank correlation coefficients were used to examine the correlation between NT levels, glycaemia and HOMA-IR. The level of significance was set at \( p < 0.05 \).

RESULTS AND DISCUSSION

The baseline clinical and metabolic characteristics of the study participants are presented in Table 1. The MetS patients had significantly higher levels of waist circumference, FG, total cholesterol, TG, systolic BP, diastolic BP and significantly lower HDL cholesterol. Serum NT levels tended to be higher in patients with MetS, although they did not differ significantly from the healthy subjects. So far, the data published about levels of circulating NT in MetS, IGT and T2DM are rather controversial. Some authors have concluded that detection of elevated plasma NT concentrations in patients with MetS [17] and T2DM [13] is sure evidence of OxS, while according to others hyperglycaemia and IGT do not affect systemic NT concentrations [18, 19]. This discrepancy could be partially explained with the age difference between the subjects in the cited investigations as well as with the age difference between patients and controls, since NT accumulates during the aging process [10, 20]. Variety of MetS comorbidities or complications could enhance protein Tyr nitration [20]. However, our age- and sex-matched study conducted newly diagnosed patients with nascent MetS, uncomplicated with T2DM or CVD, and has sufficient power to detect even small differences of NT levels.

Majority of the studies [13, 14, 17–19] have examined NT levels in blood plasma, but the difference in fibrinogen levels have not been taken into account. This acute phase protein is one of the principal targets for Tyr nitration, so a myriad of pro-thrombotic and inflammation-related conditions can augment fibrinogen levels, thus increasing the total concentration of protein-bound NT [21, 22]. Therefore, we detected NT in serum samples.

Table 1. Biochemical and anthropometric characteristics of the study groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with MetS (n=63)</th>
<th>Control subjects (n=34)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, Male/Female (no.)</td>
<td>28/35</td>
<td>17/17</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.5 ± 11.6</td>
<td>41.2 ± 10.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>97 ± 14</td>
<td>83 ± 10</td>
<td></td>
</tr>
<tr>
<td>Serum glucose (mmol/L)</td>
<td>5.25 ± 0.98</td>
<td>4.7 ± 0.41</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.39 ± 1.67</td>
<td>3.68 ± 0.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.18 (1.1–1.26)</td>
<td>1.35 (1.28–1.41)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.63 ± 0.71</td>
<td>1.24 ± 0.31</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>120 ± 11</td>
<td>118 ± 6</td>
<td>0.012</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>86 ± 4</td>
<td>79 ± 5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.34 (1.51–3.59)</td>
<td>Not assessed</td>
<td></td>
</tr>
<tr>
<td>NT (nmol/L)</td>
<td>12.59 (3.79–22.68)</td>
<td>4.23 (1.73–18.32)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or median (25th–75th percentile).
Since OxS and nitroxidative stress are caused by imbalance between ROS/RNS and antioxidants, ameliorated antioxidant defense could counterbalance increased nitroxidative stress in MetS, accounting for the non-significant increase of NT levels. Bo et al. reported elevated plasma NT levels only in diabetic patients with lower than recommended daily intake of antioxidant vitamins C and A [19]. Recent studies on MetS have confirmed the hypothesis that in response to aggravated OxS, cells upregulate the primary antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase and catalase, thus attempting to prevent oxidative injury [23, 24]. On the other hand inactivated through Tyr nitration manganese SOD has been detected in acute and chronic inflammatory processes both in animal models and in humans [25]. Nevertheless, antioxidant status of the research subjects have not been assessed in some of the above-mentioned investigations [14, 17, 18], demonstrating increased NT levels in MetS and T2DM. This was also a limitation of our research.

Although we did not find a significant difference in serum NT levels between the patient and the control groups, NT concentrations significantly and progressively increased with the increase of MetS components. Subjects who fulfilled all the five diagnostic criteria for MetS [1] had significantly higher NT levels, compared both to controls and patients with only three MetS components (Table 2).

These results are in accordance with the study, conducted by Yubero-Serrano et al. Yet, this research group did not include healthy controls, but compared only MetS patients with varying from two to number of MetS components [24]. Any of the MetS components by itself can cause over-activation of NADPH oxidase, increased production of ROS and superoxide radicals (O$_2^•−$), in particular [3]. Under conditions of ROS surplus, •NO can be oxidatively inactivated though a diffusion-controlled reaction with O$_2^•−$, generating the powerful oxidizing and nitrating agent peroxynitrite (ONOO−) [10, 26]. The production of the short-lived ONOO− (half-life ca. 10 ms) [26] can be indirectly inferred by the presence of the stable NT. Our results of a significant increase of NT levels only in the cohort with 5 MetS components confirmed that MetS is not merely a cluster of risk factors, but its components can interact and amplify the effect of each other. In addition, the formation of peroxynitrite and NT also leads to a loss of the beneficial anti-inflammatory, anti-proliferative and anti-aggregant actions of •NO, well-known as the major determinant of the normal homeostasis of cardiovascular (CV) system [11]. Thus the progressive increase of NT concentrations in parallel with the number of MetS components suggests a strict association between nitroxidative stress and common CV and metabolic burden.

Furthermore, a positive correlation between serum NT and fasting glucose was established only in the presence of all five MetS components (Fig. 1) which also favored the hypothesis for the profound complexity of MetS. Despite the fact that acute hyperglycaemia has been shown to induce NT overproduction, even in the plasma of healthy subjects [27], we observed that the harmful effect of chronic

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**Table 2. Serum nitrotyrosine (NT) levels according to the number of MetS components**

<table>
<thead>
<tr>
<th>Measured serum biomarker</th>
<th>Control subjects (n = 34)</th>
<th>Patients with MetS (n = 63)</th>
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<tr>
<td></td>
<td></td>
<td>Subjects with 3 MetS components (n = 17)</td>
</tr>
<tr>
<td>NT (nmol/L)</td>
<td>4.23 (1.73–18.32)</td>
<td>6.24 (1.8–9.8)</td>
</tr>
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</table>

Data are presented as median (25th–75th percentile). $^a$ Compared to the control subjects, $p<0.0001$; $^b$ Compared to the subjects with 3 components of MetS, $p<0.0001$.  

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hyperglycaemia could only be displayed on the background of the other four MetS features.

Accumulation of MetS components is associated with nitroxidative stress, which may impair the insulin-stimulated glucose uptake in insulin-sensing tissues like liver, muscles and adipose tissue. *In vitro* investigations have shown that ONOO− increases nitration of insulin receptor – beta (IR-β), insulin receptor substrate (IRS)-1, IRS-2 and Akt in muscles [28]. Moreover, Tyr nitrination has been associated with hepatic IR and disturbed glucose metabolism regulation in a lipid infused mice model [29]. Recent research has also demonstrated glucose-mediated elevation of Tyr nitrination in adipocytes [30]. Furthermore, production of NT reduces •NO bioavailability. At physiological levels •NO acts as a signaling molecule regulating energy homeostasis in adipose tissue by stimulating glucose uptake and insulin-responsive glucose transporter protein-4 (GLUT4) translocation along with increasing glucose and fatty acid metabolism [31]. Thus Tyr nitrination has been associated with the onset of IR and subsequent development of compensatory hyperinsulinemia. The full transition to overt T2DM is triggered by β-cell failure. Interestingly, elevated NT-staining in islets from diabetic mice has been related to protein oxidation damage and death of pancreatic β-cells [32].

Considering these data as well as the central role of visceral obesity in the development both of MetS and IR, we hypothesized that nitroxidative stress may be a key link between metabolic abnormalities in MetS and underlying IR. To investigate this suggestion we also evaluated the association between NT levels and insulin sensitivity. Nitrotyrosine concentrations positively correlated with fasting insulin levels and hence with HOMA-IR within the general MetS population (Fig. 2). There was not a significant difference in the magnitude of this correlation between MetS subgroups according to the number of MetS components (data not shown).

The observed weak, but significant positive correlation might indicate that nitroxidative stress alters the intracellular signaling pathways by inducing hyperinsulinemia and IR. This may also be associated with the second, MPO-dependent pathway for Tyr nitrination [10, 26] and the increased activity of MPO in obesity-related conditions [33]. MPO is an enzyme expressed abundantly in granules of neutrophils and to a lesser extent in monocytes, the first cells responding to an inflammatory challenge [34]. Therefore, MPO is usually associated with OxS, chronic and acute inflammation. In MetS patients, neutrophils have been correlated with HOMA-IR and the prototypic biomarker of inflammation, high sensitivity C-reactive protein (hsCRP) [35]. Furthermore, serum MPO has also been associated with IL-6 in impaired fasting glucose [36]. Consequently, Heinecke et al. proposed MPO as a mediator of IR [37]. This delineates the possible role of MPO-nitrination as another mechanism that links increased protein Tyr nitrination, chronic subclinical inflammation and IR in MetS.

In addition, peroxinitrite can be directly cytotoxic for endothelial cells and NT has been proposed as a biomarker of diabetic macro-and microvascular complications [38]. MPO has also emerged as a widely used marker for CV risk [11, 33, 39], especially on the background of existing IR and T2DM [40]. Furthermore, a strong correlation between circulating protein NT and the severity of CAD has been recently documented. In the same study, treatment with statins, well known indirect antioxidants, has diminished NT levels [15]. Although larger clinical trials are needed to confirm this finding, it has also suggested the promising role of NT in monitoring vasculoprotective and antioxidant therapies.

**CONCLUSIONS**

The current study demonstrates a positive correlation between nitroxidative stress, manifested by serum NT concentrations, and insulin resistance, measured by HOMA-IR, in subjects with nascent MetS. This finding suggests possible involvement of nitroxidative stress in MetS pathogenesis, since insulin resistance has been previously recognized as the major underlying mechanism in the develop-
ment of the syndrome. Furthermore, subjects who fulfill all the five diagnostic criteria for MetS may be exposed to a higher level of nitroxidative stress and its detrimental effects. This statement is also supported by the positive correlation between NT and fasting glucose only in the cohort with five MetS components. Therefore, the measurement of serum nitrotyrosine in subjects with metabolic syndrome might contribute to the identification of a subset of patients at increased risk of metabolic and cardiovascular complications.

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Метаболитният синдром (МС) представлява комплекс от метаболитни нарушения, включващ централно затлъстяване, хипертензия, хипертриглицеридемия, намалени нива на липопротеиновите комплекси с висока плътност (HDL холестерол) и хипергликемия. Натрупват се все повече доказателства за тясната връзка между „нитроокислителния“ стрес с МС и неговите метаболитни и сърдечносъдови усложнения. „Нитроокислителният“ стрес може да се оцени чрез нивото на 3-нитротирозин (НТ), който е стабилен продукт на посттранслационна модификация на белтъци. Ограничени и доста противоречиви са изследванията върху серумните концентрации на НТ при МС. Затова целта на настоящото проучване е да определи серумните нива на НТ при пациенти с неусложнен МС, както и да изясни връзката между НТ концентрации с нивото на глюкоза на гладно и с инсулиновата чувствителност, оценена чрез хомеостазния модел на инсулинова резистентност (HOMA-IR).

Изследвани са 63 пациенти с МС и 34 здрави контроли. Серумните концентрации на НТ са определени чрез ELISA метод. Не се установява статистически значима разлика в концентрациите на НТ между пациентите с МС [12.59 (3.79–22.68) nmol/L] и здравите контроли [4.23 (1.73–18.32) nmol/L; \( p = 0.08 \)]. Въпреки това пациентите, при които са изпълнени петте диагностични критерии за МС, показват значително по-високи НТ нива \([28.13 (19.27–38.58) \text{ nmol/L}; n = 20]\), спрямо пациентите само с 3 МС компонента \([6.24 (1.8–9.8) \text{ nmol/L}; n = 17, \ p < 0.0001]\) и здравите контроли \(p < 0.0001\). Положителна корелация между серумните нива на НТ и глюкоза се доказва само при наличие и на петте компонента на МС \(n = 20, r = 0.485, p = 0.03\), докато корелацията с HOMA-IR индекса е валидна за цялата пациентска група \((r = 0.287, p = 0.025)\).

„Нитроокислителният стрес“ може би е свързан с патогенезата на инсулиновата резистентност при МС и се увеличава пропорционално с нарастването на броя на компонентите на МС.