Research Article

Andrada Mihai, Daniela Lixandru*, Petruta Alexandru, Irina Stoian, Simona Carniciu, Manuela Mitu, Cristian Guja, Constantin Ionescu-Tirgoviste

Negative association between paraoxonase 2, anthropometric markers and metabolic syndrome

DOI 10.1515/biol-2015-0037
Received January 18, 2015; accepted May 11, 2015

Abstract: Background Metabolic syndrome (MS) has a great impact on cardiovascular mortality and morbidity. Our aim was to investigate the association of MS with some oxidant and antioxidant markers, including pro-and antioxidant status of peripheral blood mononuclear cells (PBMC) in newly diagnosed type 2 diabetic mellitus patients (ND-T2D).

Methods 219 ND-T2D and 88 healthy subjects were divided in two groups according to the absence or presence of MS. Anthropometric measurements, routine blood tests, total oxidant status (TOS), total antioxidant status (TAS) and ELISA measurements were included. The PBMC capacity to release free radicals and to neutralize them was also determined by measuring the respiratory burst (RB) together with the lactonase activity of the intracellular antioxidant enzyme paraoxonase 2 (PON2).

Results Comparing ND-T2D MS+ with those MS- the RB of the PBMC was significantly higher (p<0.05) while lactonase PON2 enzymatic activity was decreased (p < 0.001). A negative correlation of RB was found with TAS (r = -0.416, p < 0.001). PON2 was also negatively correlated with glycaemia (r = -0.275, p < 0.001), HbA1c (r = -0.308, p < 0.001), weight (r = -0.183; p < 0.05), waist circumference (r = -0.353, p < 0.001) and body mass index (r = -0.290, p < 0.001).

Conclusion PON2 lactonase activity is negatively associated with anthropometric markers in ND-T2D with MS.

Keywords: type 2 diabetes, metabolic syndrome, paraoxonase 2, total oxidant and antioxidant status

1 Introduction

Metabolic syndrome (MS), a collection of cardiometabolic risk factors, invariably associated with obesity, central distribution of body fat, dyslipidemia and hypertension [1,2] is often characterized by oxidative stress, an imbalance between the production and inactivation of reactive oxygen species [3].

Unlike Paraoxonase 1 (PON1) and PON3 that are associated with high-density lipoproteins (HDL) in plasma [4], PON2 is not linked to HDL being an intracellular enzyme expressed ubiquitously [5,6]. All three PONS are antioxidant enzymes and lactonases, but PON2 displays the highest activity hydrolyzing a number of acyl-homoserine lactones [7,8].

Therefore, in the development of microvascular and macrovascular complications of type 2 diabetes (T2D) the disturbance of oxidant-antioxidant balance plays an important role [9]. Taking these into account by lowering the intracellular or local stress of a cell, PON2 is able to prevent the oxidation of low density lipoproteins (LDL), protecting lipoproteins from oxidative modifications [5].

Increase in oxidative stress due to persistence in the circulation of triglyceride-rich lipoproteins as chylomicrons (CM) and very low density lipoproteins (VLDL), together with hyperglycemia [10] is intricately linked to alterations in Monocyte/Macrophage (Mo/M GBP) function [11]. Release of free radicals by the respiratory burst (RB) of peripheral blood mononuclear cells (PBMC) increases in diabetes and is proportional to the levels of glucose and triglycerides in the blood [12,13]. Increased peroxidation of lipoproteins contributes to foam cell formation by facilitating lipid uptake into macrophages [14] and is also potentially modulated by the intracellular...
antioxidant capacity of the Mo/Mø, which is mainly ensured by antioxidant enzymes and could thus delay atherosclerosis [15,16].

We aimed to investigate the impact of metabolic syndrome on some oxidant and antioxidant markers, including pro-and antioxidant status of peripheral blood mononuclear cells in newly diagnosed type 2 diabetic patients (ND-T2D).

2 Methods

219 newly (within 6 months) diagnosed patients, 115 females (mean age 61 ± 10 years) and 104 males (mean age 57 ± 10 years) with type 2 diabetes from outpatients treated at the National Institute of Diabetes, Nutrition and Metabolic Disease (NIDNMB) “Prof. N.C. Paulescu” were selected. The study was approved by the Ethics Committee of NIDNMB “Prof. N. Paulescu” and all participants signed informed consent before inclusion in the study.

Diabetes was diagnosed according to the WHO (World Health Organisation) criteria: serum fasting glucose ≥ 7.0 mmol/L (126 mg/dL), or 2 hour serum glucose obtained after an oral glucose tolerance test (OGTT) ≥ 11.1 mmol/L (200 mg/dL), or an HbA1c ≥ 49 mmol/mol or a random plasma glucose ≥ 11.1 mmol/L (200 mg/dL) in patients accompanied by classical symptoms such as polyuria, polyphagia, polydipsia as well as a weight loss [17,18]. The diabetic group was divided into two subgroups according to the presence (MS+) or absence (MS-) of metabolic syndrome (MS) diagnosed according of consensus definition [19] with three or more of the following criteria included: (i) increased waist circumference (WC): ≥ 94 cm for men and ≥ 80 cm for women, (ii) elevated triglycerides: ≥ 1.70 mmol/L, or specific treatment for this lipid abnormality, (iii) decreased HDL-cholesterol: < 1.03 mmol/L in men, < 1.30 mmol/L in women, or specific treatment for this lipid abnormality, (iv) increased systolic blood pressure ≥ 130 and/or diastolic ≥ 85 mmHg, or antihypertensive drug treatment in patient with history of hypertension, and (v) increased fasting glucose > 5.55 mmol/L, or drug treatment of increased glucose.

88 healthy subjects, 30 females (mean age 53 ± 12 years) and 58 males (mean age 49 ± 14 years) were recruited as control group, without any suspicion of diabetes or glucose intolerance (normal glycemia as well as HbA1c < 44 mmol/mol).

The exclusion criteria for all subjects were: hemodialysis treatment, acute ischemic cardiovascular disease, hemorrhagic stroke, urinary infections, epilepsy or other severe diseases (e.g. hepatic failure, cancer or gangrene) and also, use of vitamins, minerals, or other supplements in the previous month, excessive alcohol consumption (ethanol > 20 g/day) and pregnant women.

After an overnight fast, blood samples were collected into vacuum tubes with no (for routine blood tests and ELISA measurements) or EDTA-containing (for isolation of PBMC, fructosamine, TAS and TOS). A volume of 10mL from each sample containing EDTA was immediately used for isolation of PBMC for Respiratory Burst (RB) and paraoxonase 2 (PON2) while serum in the remained sample (10 mL) after centrifugation was stored at -80°C until required.

Antioxidant measurements included: weight, height, Body Mass Index (BMI; kg/m²) and visceral fat (%) assessed using a bioelectrical impedance analyzer (Body Composition Analyzer MC-980 TANITA).

Routine blood tests including the concentration of fasting glucose, Hba1c, total cholesterol, HDL-cholesterol (“high density lipoprotein”; HDLc), total triglycerides (TG), urea, creatinine and uric acid were measured using current biochemical methods on a Hospitex Diagnostincs Eos Bravo Forte Analyser. Serum concentrations of insulin, proinsulin, C peptide, leptin and adiponectin were determined by ELISA method on Multiskan ExThermo Electro Corporation using commercially available kits (EIA-2935, EIA-1560, EIA-1293, EIA-2395 and respectively EIA-4177; DRG Instruments GmbH, Germany) following the manufacturer’s guidelines. The coefficient of variation (CV) was respectively 2.2%, 4.86%, 6.12%, 6.43% and 5.66%. LDL-cholesterol (“low density lipoprotein”; LDLc) was calculated according to the Friedewald equation [20] and HOMA-IR (“Homeostasis Model for Insulin Resistance”) was recorded as [glycemia (mmol/L) × insulinemia (μU/mL)] / [22.5] [21].

Fructosamine was done by using the CAIMAN kit (CV = 5%) using a colorimetric assay method based on the ability of ketoamines to reduce nitrotetrazolium-blue (NBT) to formazan, measured photometrically at 546 nm in an alkaline solution. Plasma total oxidant status (TOS) and total antioxidant status (TAS) were done using the kits no. KC5100 (CV = 2.94%) and no. KC5200 (CV = 3.99%) respectively, both photometric tests from Immundiagnostik.

Isolation of peripheral blood mononuclear cells (PBMC) and measurement of Respiratory Burst (RB): PBMC were isolated by density centrifugation on Ficoll-Paque™ Plus (1.0077 g/mL) at 630 g for 30 minutes. Cell viability by Trypan Blue exclusion was ≥ 90%. The ability to produce a RB was monitored by luminol-enhanced chemiluminescence method [22]. In short, to PBMC (0.3
× 10⁶ cells) washed twice and resuspended in 1mL PBS, dark-adapted luminal was added. After monitoring spontaneous chemiluminescence for 15 min, the RB was initiated by adding of 100 µL phorbol 12-myristate 13-acetate (PMA) (final concentration 5.4 µmol/L) or opsonised Zymosan (ZO) (final concentration 0.83 g/L) and the maximum chemiluminescence peak was recorded (Luminoskan Ascent® 392, LabsystemsEx-Thermo Electro Corporation). Chemiluminescence production was expressed as the Relative Chemiluminescence Units over time (RLUX60min).

Paraoxonase 2 lactonase activity was measured towards dihydrocoumarin at 270nm using a continuously recording CECIL-CE 1010 UV/VIS spectrophotometer. Protein concentration was determined using the method of Bradford [23]. PBMC cells (30 × 10³) after collection were washed (2X) with PBS and enzyme activity was measured using 1000 µg protein/mL by adding 100 µL cells to 1900 µL Tris buffer (25 mmol/L Tris/HCl, 1 mmol/L CaCl₂, pH 7.6) containing 1mmol/L DHC. One unit of lactonase activity is equal to 1 µmol of DHC hydrolyzed/mL/min using the extinction coefficient of 1295 M⁻¹cm⁻¹ [24].

All reagents, Dulbecco’s Phosphate Buffered Saline (PBS), Fetal Bovine Serum (FBS), Ficoll-Paque™ Plus, Trypan Blue, phorbol 12-myristate 13-acetate (PMA), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; LM), Zymosan A from Saccharomyces cerevisiae and dihidrocoumarine (DHC) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.1 Statistical Analysis

Differences between groups were analyzed with Epi Info 7.1.0.6 statistical software (Centre for Disease Control, Atlanta) using Student t-test on independent samples. Correlations between parameters were evaluated by the Pearson test. A value for p < 0.05 was considered statistically significant. Testing the normality of the distribution of variables was performed using the Kolmogorov-Smirnov test. The variables without a normal distribution were log-transformed to normalize the distribution and subsequently analyzed. For uniformity, results were expressed as mean ± standard deviation (SD)/standard error of the mean (SEM) for all parameters.

3 Results

The clinical characteristics and routine biochemistry of the healthy subjects and diabetic patients are shown in Table 1. Notably, there was no significant difference in the ages of T2D patients and control subjects. Comparing people with T2D MS- with those MS+ for anthropometric markers we found that weight, waist circumference and BMI were decreased in the first group (p < 0.001). Also, serum uric acid and ALT levels were decreased when comparing MS- with MS+ but only in the T2D group.

With regard to ELISA results, control subjects (MS-) and (MS+) had no differences in insulin, proinsulin, C peptide and leptin levels while all were significantly higher in T2D group MS+. As expected, people with T2D and MS+ also had lower adiponectin concentrations (Table 2).

TOS results in the T2D group MS+ and MS- are presented in Figure 1A while TAS results for the studied T2D groups are presented in Figure 1B. Figure 2 presents the results obtained for Respiratory Burst (Figure 2A) and lactonase PON2 enzymatic activity (Figure 2B) in patients with T2D with or without MS.

4 Discussion

Several studies mentioned the components of MS, glucose intolerance and obesity, as causing changes of oxidative stress markers [25-28]. Moreover, it has been shown that measurements of abnormalities in oxidative status in PBMC cells comparing to total oxidant/antioxidant capacity could be a useful indicator of the progression of the disease related with accelerated atherogenesis [10].

In this study, we analyzed TOS and TAS as the overall markers of oxidative stress and the pro-and antioxidant status of peripheral mononuclear cells (PBMC) by measuring the RB and PON2 lactonase activity, respectively, in ND-T2D patients. Out of the anthropometric markers studied, we found that both WC and BMI were most correlated with PON2 enzymatic activity. It is notable that with the increased numbers of MS criteria PON2 lactonase activity in Mo/Mₒ decreased.

TOS was increased (p < 0.001) in the MS+ group with T2D (Figure 1A), but not in MS+ group without diabetes. There was no significant difference between the two groups in TAS (Figure 1B). Respiratory Burst of PBMC was significantly higher in patients with T2D and MS+ (p < 0.05) (Figure 2A), while lactonase PON2 enzymatic activity was decreased (p < 0.001) (Figure 2B). For diabetes, a negative correlation of TAS was found with RB LM/PMA (r = -0.416, p < 0.05).

In this study, PON2 was negatively correlated with weight (r = -0.183; p < 0.05), with WC (r = -0.353, p < 0.001) and BMI (r = -0.290, p < 0.001). Moreover, the PON2 was negatively and TOS positively correlated with
A. Mihai et al.

the number of metabolic syndrome criteria, $r = -0.406$ ($p < 0.001$) and $r = -0.280$ ($p < 0.05$) respectively. PON2 was found to be negatively correlated with glycaemia ($r = -0.275$, $p < 0.001$), HbA1c ($r = -0.308$, $p < 0.001$), HOMA-IR ($r = -0.204$, $p < 0.05$), proinsulin ($r = -0.165$, $p < 0.05$) and C-peptide ($r = -0.180$, $p < 0.05$). For controls, proinsulin was found to be negatively correlated with RB ($r = -0.272$, $p < 0.05$) and C-peptide with PON2 ($r = -0.250$, $p < 0.05$). Insulin positively correlated with leptin ($r = 0.167$, $p < 0.05$) and negatively with adiponectin ($r = -0.123$, $p < 0.05$). No correlation was found between RB and leptin or adiponectin.

In a recent paper, Savu et al. showed that patients with uncomplicated T2D improved their total antioxidant capacity of plasma despite high levels of oxidative stress [29].

### Table 1. The clinical and biochemical characteristics of T2D versus healthy subjects.

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>T2D (n = 27)</th>
<th>MS+ (n = 192)</th>
<th>Healthy Subjects (n = 70)</th>
<th>MS+ (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58 ± 10</td>
<td>59 ± 10</td>
<td>50 ± 14</td>
<td>52 ± 10</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>76.96 ± 13.42**</td>
<td>90.32 ± 16.11</td>
<td>72.52 ± 15.60**</td>
<td>100.46 ± 13.55</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>95.88 ± 12.03**</td>
<td>107.94 ± 10.85</td>
<td>90.32 ± 16.11</td>
<td>107.25 ± 12.12</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.30 ± 3.47**</td>
<td>32.20 ± 4.76</td>
<td>26.16 ± 4.87**</td>
<td>32.80 ± 3.04</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126.67 ± 10.76</td>
<td>130.86 ± 10.85</td>
<td>121.78 ± 13.42**</td>
<td>132.86 ± 11.12</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>71.30 ± 8.61</td>
<td>74.62 ± 7.82</td>
<td>73.49 ± 10.53**</td>
<td>84.29 ± 5.34</td>
</tr>
<tr>
<td>Glycemia (mg/dL)</td>
<td>183.78 ± 67.72</td>
<td>176.31 ± 69.52</td>
<td>94.59 ± 14.90</td>
<td>104.25 ± 15.87</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.02 ± 2.15</td>
<td>7.81 ± 1.96</td>
<td>5.88 ± 0.22</td>
<td>5.69 ± 0.03</td>
</tr>
<tr>
<td>Serum total cholesterol (mg/dL)</td>
<td>214.68 ± 46.72</td>
<td>220.49 ± 56.32</td>
<td>211.51 ± 46.77</td>
<td>227.07 ± 40.76</td>
</tr>
<tr>
<td>Triglycerides (TG) (mg/dL)</td>
<td>103.51 ± 49.56**</td>
<td>182.12 ± 107.28</td>
<td>96.90 ± 47.32**</td>
<td>176.55 ± 58.18</td>
</tr>
<tr>
<td>Serum LDLc (mg/dL)</td>
<td>141.74 ± 40.97</td>
<td>139.81 ± 49.61</td>
<td>143.52 ± 46.03</td>
<td>150.75 ± 31.76</td>
</tr>
<tr>
<td>TG/HDLc</td>
<td>2.05 ± 0.95**</td>
<td>4.68 ± 3.75</td>
<td>2.12 ± 1.74**</td>
<td>4.39 ± 1.70</td>
</tr>
<tr>
<td>AST (U/mL)</td>
<td>22.37 ± 11.61</td>
<td>25.03 ± 15.77</td>
<td>21.02 ± 7.99</td>
<td>17.88 ± 5.60</td>
</tr>
<tr>
<td>ALT (U/mL)</td>
<td>24.54 ± 10.41**</td>
<td>32.63 ± 21.87</td>
<td>20.32 ± 11.42</td>
<td>23.91 ± 15.65</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.75 ± 0.17*</td>
<td>0.84 ± 0.16</td>
<td>0.88 ± 0.18</td>
<td>0.94 ± 0.15</td>
</tr>
<tr>
<td>Uric Acid (mg/dL)</td>
<td>4.42 ± 1.59**</td>
<td>5.77 ± 1.85</td>
<td>4.58 ± 1.71</td>
<td>5.08 ± 1.29</td>
</tr>
<tr>
<td>GGT (U/mL)</td>
<td>37.18 ± 43.46</td>
<td>47.06 ± 36.56</td>
<td>26.25 ± 18.21</td>
<td>37.28 ± 43.95</td>
</tr>
<tr>
<td>Fructosamine (µmol/l)</td>
<td>557.23 ± 204.49</td>
<td>566.64 ± 257.82</td>
<td>403.64 ± 105.04</td>
<td>406.75 ± 106.46</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4.54 ± 2.76**</td>
<td>6.53 ± 7.90</td>
<td>1.99 ± 1.43</td>
<td>3.20 ± 2.03</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. * and ** denotes $p < 0.05$ and $p < 0.001$ when comparing MS- to MS+ within different groups; SBP – systolic blood pressure; DBP – diastolic blood pressure; AST – aspartat aminotransferase; ALT – alanine aminotransferase; GGT – gamma glutamyltransferase; HOMA-IR - Homeostasis Model Assessment for Insulin Resistance;

### Table 2. The results of ELISA measurements for the T2D versus healthy subjects.

<table>
<thead>
<tr>
<th>PA PARAMETERS</th>
<th>T2D (n = 27)</th>
<th>MS+ (n = 192)</th>
<th>Healthy Subjects (n = 70)</th>
<th>MS+ (n = 18)</th>
<th>p-value (T2D vs. Healthy Subjects)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (µIU/ml)</td>
<td>9.90 ± 1.92**</td>
<td>14.52 ± 0.71</td>
<td>8.51 ± 0.67</td>
<td>12.22 ± 2.59</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Proinsulin (pmol/l)</td>
<td>5.01 ± 1.06**</td>
<td>7.45 ± 0.58</td>
<td>2.43 ± 0.19</td>
<td>2.72 ± 0.94</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>C-peptide (ng/mL)</td>
<td>3.33 ± 0.38**</td>
<td>5.01 ± 0.25</td>
<td>2.93 ± 0.17</td>
<td>4.08 ± 0.73</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>9.39 ± 1.64**</td>
<td>17.43 ± 1.14</td>
<td>16.80 ± 2.04</td>
<td>13.27 ± 5.03</td>
<td>NS</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>10.71 ± 2.09</td>
<td>7.39 ± 0.53</td>
<td>14.76 ± 1.30**</td>
<td>8.49 ± 1.27</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Proinsulin/insulin</td>
<td>0.54 ± 0.10</td>
<td>0.55 ± 0.04</td>
<td>0.33 ± 0.02</td>
<td>0.30 ± 0.08</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Proinsulin/adiponectin</td>
<td>1.67 ± 0.63</td>
<td>2.32 ± 0.29</td>
<td>0.32 ± 0.04</td>
<td>0.39 ± 0.16</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. * and ** denotes $p < 0.05$ and $p < 0.001$ when comparing MS- to MS+ within different groups; NS = not significant;
of cellular antioxidant enzymes, as the PON2, may have a crucial role. The PON2 enzyme has been known for many years as an intracellular membrane-associated protein that protects against oxidative stress [5,30] and in our study was found to be decreased in MS+ group. This could suggest a close relationship between intracellular ROS and antioxidant enzyme PON2 in PBMC, increased oxidative stress being associated with metabolic syndrome as a hallmark of the patient that often develops atherosclerosis.

5 Conclusion

Our study highlights the prooxidant/antioxidant imbalance in PBMC from diabetic patients with metabolic syndrome. PON2 low activity and increased NADPH oxidase activity in PBMC is influenced by MS presence. Oxidative stress induced by hyperglycemia associated with the presence of MS can be one of the risk factors that occur early in the natural history of diabetes. Further studies are necessary to validate early oxidant/antioxidant biomarkers even before the onset of hyperglycemia, with the development of potential non-invasive therapies to improve diabetic condition.

Acknowledgments: This work was supported by a grant from the Romanian National Authority for Scientific Research, CNCS-UEFISCDI, project number PN-II-IDPCE-2011-3-0429. Dr. Daniela Lixandru was also supported by the postdoctoral program POSDRU/89/1.5/S/60746 and Petruta Alexandru by the program POSDRU/107/1.5/S/82514 from European Social Fund. We gratefully acknowledge Laura Petcu, Ariana Picu and Janeta Tudosoiu from the research laboratory of NIDNMD “Prof. N. Paulescu”, for excellent technical assistance in conducting the clinical study.

Conflict of interest: Dr Lixandru has nothing to disclose.

References


[28] Bacanu E., Lixandru D., Stoian I., Virgolici B., Mohora M., Mitrea N., Ionescu-Tirgoviste C., Correlations between obesity antropometric markers, adipocytokines and monocytes oxidative stress status in type 2 diabetic patients, Farmacim, 2012, 60 (1), 21-32

[30] Reddy S.T., Devarajan A., Bourquard N., Shih D., Fogelman A.M., Is it just paraoxonase 1 or are other members of the paraoxonase gene family implicated in atherosclerosis? Curr. Opin. Lipidol., 2008, 19, 405-408