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INFLAMMATION MARKERS IN PATIENTS WITH CARDIOVASCULAR DISEASE AND METABOLIC SYNDROME

MARKERI INFLAMACIJE KOD PACIJENATA SA KARDIOVASKULARNOM BOLEŠĆU I METABOLIČKIM SINDROMOM

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Summary

Background: The clinical value and the interrelationship of HDL and the metabolic syndrome were studied using plasma levels of TNF-α, IL 6, IL 10, IL 8, IL 1beta, IL 2R in patients with cardiovascular stenosis.
Methods: On the basis of exclusion criteria, we recruited 198 male and female patients aged 45 to 75 years with CVD and 43 patients with MS. Patients were subdivided into %stenosis according to the CASS guidelines. Lipids were measured on an Olympus AU640 analyzer. Ox-LDL was measured by the immunosorbent assay and MDA by HPLC. Cytokines were analysed with DPC Immulite 1000. Statistical tests were performed using SPSS for Windows, 14.0 & Medcalc.
Results: Ox-LDL and apoB were significantly higher in the MS(+) patient group (88.7 U/L) compared to the MS(-) group (77.5 U/L). Ox-LDL showed a positive correlation (P=0.001) with LDL-C, apoB and MDA. There was a higher concentration of HDL in the patient group MS(-), which was confirmed by a non-significant (P=0.849) change of apoA(I) from 1.267 g/L in the MS(+) to 1.275 g/L in the MS(-) group. A light significant increase of IL 10 (P=0.05) in MS(+) patients was observed, and the other analysed inflammation markers were mostly unchanged. MS has no direct association with the cytokine production.
Conclusions: Ox-LDL and apoB were significantly higher in the MS(+) patient group. In a multiple regression analysis for

Kratak sadržaj

Uvod: Kod pacijenata sa kardiovaskularnom stenozom, proučavana je klinička vrednost kao i međusobni odnos HDL i metaboličkog sindroma, pomoću nivoa TNF-α, IL 6, IL 10, IL 8, IL 1beta, IL 2R.
Metode: U skladu s kriterijumima za isključenje, obuhvaćeno je 198 pacijenata, muškog i ženskog pola, starosti između 45 i 75 godina, sa kardiovaskularnom bolešću i 43 pacijenta sa metaboličkim sindromom. Pacijenti su dalje podeljeni prema procentu stenoze na osnovu preporuka CASS. Lipidi su mereni na analizatoru OLYMPUS AU640. Za merenje ox-LDL korišćen je test ELISA a za MDA metoda HPLC. Citokini su analizirani na uređaju DPC IMMULITE 1000. Za statističku obradu podataka upotrebljen je SPSS za Windows, 14.0 i Medcalc.
Rezultati: Ox-LDL i apo-B bili su značajno viši u grupi pacijenata MS(+) (88,7 U/L) u poređenju s grupom MS(-) (77,5 U/L). Ox-LDL bio je u pozitivnoj korelaciji (P=0,001) sa LDL-C, apo-B i MDA. U grupi MS(-) pronađena je veća koncentracija HDL, što je potvrdila i nesignifikantna promena (P=0,849) apo A(I) od 1.267 g/L u grupi MS(+) do 1.275 g/L u grupi MS(-). Neznatan ali značajan porast IL 10 (P=0,05) uočen je kod pacijenata MS(+), dok su ostali analizirani markeri inflamacije uglavnom bili nepromenjeni. MS nije direktno povezan s proizvodnjom citokina.
Zaključak: Ox-LDL i apo-B bili su značajno viši u grupi pacijenata MS(+). Analiza višestruke regresije za ox-LDL ukaza-

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ox-LDL, apoB (P=0.003) emerged as a strong predictor of the ox-LDL concentration, independent of age, gender, BMI and smoking.

Keywords: metabolic syndrome, inflammation, oxidative stress, stenosis, HDL lipoproteins

la je na apo-B (P=0,003) kao na jak prediktor koncentracije ox-LDL, nezavisan od starosti, pola, indeksa telesne mase i pušenja.

Ključne reči: metabolički sindrom, inflamacija, oksidativni stres, stenoza, HDL lipoproteini

Introduction

Atherosclerosis is no longer only a lipid disorder, but rather a process of dynamic interactions between endothelial dysfunction, subendothelial inflammation and the wound healing response of the vascular smooth muscle cells. As we studied the influence of the degree of stenosis on the biological markers, a new classification of atheromatous lesions is described. The first step is the intima thickened by loose, lipid-rich, fibrous tissue with no lipid core, followed by a multilayered cap of fibrous tissue and finalized in a lipid core with a thin inflamed fibrous cap »<80 μm in coronary and <200 μm in carotid arteries«. In a further step a rupture of the fibrous cap can occur with associated thrombus formation. In the next phase, lesions with fibrosis and large areas of calcification are observed, and at the end stages of the atherosclerotic process heavy lesions are detected (1).

Recent investigations of atherosclerosis have focused on inflammation, providing new insight into the mechanism of the disease (2, 3). The metabolic syndrome increases the risk of cardiovascular disease and type 2 diabetes (4, 5). The National Cholesterol Education Program – Adult Treatment Panel III (NCEP-ATP III) has recognized the metabolic syndrome as a cluster of abnormalities, increasing the risk of both cardiovascular disease (CVD) and type 2 diabetes (6). The NCEP-ATP III guidelines have also underlined the central role of abdominal obesity in the development of this syndrome (7). One of the major risk factors in the metabolic syndrome is the dyslipidemia which can be related to a changed lipoprotein spectrum and to modified lipoproteins (8). Chronic infection creates a proinflammatory state which is characterized by long-term elevation of cytokines and acute phase proteins and the conditions which are associated with the clinical outcome and manifestations of atherosclerotic disease (9). Increased plasma levels of »CRP, SAA, IL-6« constitute an inflammatory signature of advanced atherosclerosis and are correlated with the extent of disease, but do not provide discriminatory diagnostic power over and above the established risk factors (10).

HDL particles are independent risk factors, are cardioprotective and life style dependent. They are preventive in CV-events and potential therapeutic targets in prevention. One of the unanswered questions is the role of HDL in the inflammation process and the role of HDL in patients with the metabolic syndrome (11, 12).

The effect of metabolic syndrome on the inflammatory biomarkers, predicting the risk of clinical events, is not well known. We followed the association of CVD stenosis and metabolic syndrome with lipid risk factors and proinflammatory biomarkers in patients with angina pectoris. We investigated the interrelationship of cytokines with the protective HDL lipoproteins.

Materials and Methods

From June 2008 till 2010, patients with angina pectoris from the Department of Cardiology of the Academic Hospital »Mother Teresa« (Tirana, Albania) were selected based on exclusion criteria (MI, heart failure, CHD > 2 years, anticoagulant therapy and antibiotic therapy since two months before). The patients underwent coronarography examination and were screened and subdivided into percentage of stenosis in accordance with CASS guidelines classifications (13). Less than 50% stenosis was considered as non-significant and more than 50% was classified as significant stenosis.

On the basis of the exclusion criteria, we recruited from the coronarography unit 198 patients, males and females, aged between 45 and 75 years, with CVD, and 43 of them were screened for metabolic syndrome (MS(+)) and 155 were MS(-). For the metabolic syndrome screening we utilized and followed the recommendations of ESC/EAS guidelines and the AHA/NHLBI scientific statement (6, 14). Three basic screening parameters were applied for the selection, TG > 1.80 mmol/L, BMI > 25 kg/m^2 and hypertension: systolic > 140 and diastolic > 90 mmHg.

A history of smoking status (smokers and non-smokers), hypertension (blood pressure > 14/9 mmHg), diabetes (glucose > 6 mmol/L), statins treatment, weight, length were taken, and physical examination was performed in each patient, by the same investigator and according to the European guidelines on cardiovascular disease from the European Society of Cardiology (15).

Venous blood was sampled in serum tubes and heparinized tubes after overnight fasting. Serum and heparinized plasma samples were obtained by centrifugation (1,000 xg, 10 min) and kept frozen at –80 °C until assayed. On each sample, analytical procedures and measurements of the different lipid biomarkers were always performed within the same day to avoid repetitive freezing and thawing of the sample.

All subjects gave their consent to storing blood for this study, which was approved by the Ethical Committee of Tirana hospital.

Methods

Serum ox-LDL concentration

Serum ox-LDL concentration was measured by an enzyme-linked immunosorbent assay (ELISA) based on a murine monoclonal antibody, mAb-4E6 (16) specific for a neo-epitope in the aldehyde-substituted lysine residues of the apolipoprotein B-100 moiety of ox-LDL (Mercodia Uppsala, Sweden). The bound ox-LDL was detected with a peroxidase-conjugated anti-apolipoprotein B antibody and a colorimetric reaction with 3,3',5,5'-tetramethylbenzidine reading at 450 nm. CV (%) is 8.3 and the detection range is between 25 and 116 U/L.

Plasma MDA concentration

Plasma malondialdehyde (MDA) was assayed using a high performance liquid chromatography (HPLC) method based on the classic thiobarbituric acid (TBA) reaction (17). An aliquot of 200 μL plasma was diluted with 750 μL H_3PO_4, 0.44 moL/L and mixed with 350 μL TBA, 42 mmol/L. After heating at 100 °C for one hour, an aliquot of 20 μL was injected into the HPLC system (Merck LaChrom, Darmstadt, Germany). The TBA-MDA adduct was separated on a reversed-phase column (NOVA-pak C18 3.9 × 150 mm, Waters, Milford, MA) and monitored by fluorescence detection (λex = 515 nm, λem = 543 nm). The column was isocratically eluted at 1 mL/min with CH_3OH/0.6% KH_2PO4 pH 6.0 (30/70 v/v). The method was calibrated using 1,1,3,3-tetraethoxypropane as standard (13). CV (%) is 6.2 and the detection limit is 0.10 μmol/L.

Other biochemical parameters

Serum concentrations of total cholesterol, HDL-cholesterol and triglycerides were determined by commercially available colorimetric-enzymatic methods on an Olympus AU640 analyser. Serum LDL cholesterol concentrations were calculated using the Friedewald formula (18). Apolipoprotein A-I (apoA-I) and apolipoprotein B (apoB) concentrations were measured by immunonephelometry on a BN Prospec nephelometer (Siemens Healthcare Diagnostics, Marburg, Germany).

We analysed the serum levels of the following cytokines: TNF-α, IL 6, IL 10, IL 8, IL 1beta, IL 2R and hs-CRP by the solid-phase chemiluminescent immunometric assay on a DPC Immulite 1000 (Siemens Deerfield, IL, USA).

Statistical analysis

Data are presented as mean ± SD. Statistical significance was examined using SPSS for Windows, 14.0 and Medcalc, version 9.3. Statistical significance was considered at the level of P = 0.050.

Results

The study population group is described under Patient identification, in *Table I*. The 198 selected patients taken up in the study were subdivided in two groups, ranking under the percent stenosis: group I has less than 50% stenosis and group II has more than 50%. A significantly higher number of patients were screened in group two (n = 126) than in group one (n = 72). The ratio of Males/Females is significantly higher (P = 0.012) in group II with > 50% stenosis and confirms the gender relationship to disease development. As expected, hypertension and diabetes are significantly (P = 0.001) higher in the patient group with > 50% stenosis and confirms earlier inclusions in the guidelines regarding blood pressure. Also, in the line of prospective studies, a higher percentage of smokers was screened in the group with the highest percentage of stenosis (14). According to the EAS and AHA guidelines (6, 14), patients were selected for the Metabolic Syndrome and from the 198 patients, 43 were MS(+) and 155 were MS(-). In *Table II*, the observed data on inflammation markers and oxidative stress markers in cardiovascular patients with the metabolic syndrome were compared with the patient group without metabolic syndrome. The main result was a significantly higher concentration of ox-LDL in the MS(+) patient

Table I Patient identification: baseline characteristics of the study population; group I »<50% stenosis«, group II »>50% stenosis«.

Patients	group I (N = 72)	group II (N = 126)	P value
Sample size	72	126	
Males/Females	54% (males)	79%	P = 0.012
Age (years)	54	59	
Weight (kg)	78.09	78.8	
Diabetes	9%	26%	P = 0.001
Hypertension	65%	75%	P = 0.001
Smoking	35%	68%	P = 0.001
Statins Treatment	80%	97%	P = 0.001
Total cholesterol (mmol/L)	4.9 ± 1.4	4.8 ± 1.2	
LDL-C (mmol/L)	3.0 ± 1.1	2.9 ± 0.9	
Triglycerides (mmol/L)	1.5 ± 0.7	1.8 ± 1.3	P = 0.123

Table II Lipid and oxidative stress markers in cardiovascular patients with (MS+) and without (MS-) the metabolic syndrome.

Lipids	MS+ (N = 43)	MS- (N = 155)	P value
MDA, μmol/L	2.84 ± 0.9	2.49 ± 1.3	P = 0.207
ox-LDL, U/L	88.78 ± 36.1	77.48 ± 31.01	P = 0.064
Apo-B, g/L	1.1470 ± 0.310	0.943 ± 0.285	P = 0.0001
HDL-C, mmol/L	1.03 ± 0.26	1.09 ± 0.22	P = 0.167
ApoA(I), g/L	1.267 ± 0.232	1.275 ± 0.234	P = 0.849
Ferritin, g/L	1.760 ± 0.248	1.700 ± 0.310	P = 0.870

Table III Inflammation markers in cardiovascular patients with the metabolic syndrome.

Cytokines (pg/mL)	MS(+)(N=43)	MS(-) (N=155)	P value
TNF-α	16.62 ± 10.5	15.45 ± 10.6	P = 0.532
IL 6	6.31 ± 2.9	6.65 ± 5.2	P = 0.699
IL 8	11.96 ± 10.00	10.95 ± 7.16	P = 0.467
IL 10	5.74 ± 6.71	4.37 ± 2.87	P = 0,051
IL6/IL10	1.48 ± 0.75	1.80 ± 1.74	P = 0.264
IL 2R	766.76 ± 317.6	762.65 ± 350.37	P = 0.947

group, which was confirmed by a significant increase in apoB (1.147 g/L versus 0.943 g/dl) (P = 0.001).

In a multiple regression analysis for ox-LDL, apoB (P = 0.003) emerged as a strong predictor of the ox-LDL concentration independent of age, gender, BMI and smoking.

As shown in *Table II*, a very important result was also observed with the protective HDL particles. HDL was slightly higher in the cardiovascular patient group without metabolic syndrome, 1.09 (mmol/L), versus 1.03 (mmol/L) in the patient group with the metabolic syndrome (P = 0.167).

To understand the role of cytokines in cardiovascular events and to learn more about the influence of the metabolic syndrome on inflammation, we followed the cytokine concentrations in both groups, patients with the metabolic syndrome (MS(+)) and MS(-) patients. We calculated a lightly higher concentration of IL 10 in MS(+) patients (P = 0.051). The other analysed inflammation markers were mostly unchanged, and the metabolic syndrome seems to have no direct association with the cytokine production. TNF-α and IL 10 are higher in MS(+) patients and the ratio of IL 6/IL 10 is lower in MS(+) (P = 0.264). The cytokines are rather more proatherogenic in the patient group MS(+). The response of the acute phase proteins in the metabolic syndrome was followed by analysing the fibrinogen concentration with an increase in MS(+) patients, 3.24 versus 3.14 g/L (P = 0.561). Oxidation of PUFA by redox-active metals such as iron (Fe[+]) is considered as the initiating step in LDL oxidation (15, 25) generating the formation of malondialdehyde (MDA). To know better the influence of metabolic syndrome on active metals, ferritin was analysed in both groups, and there was no significant difference (1.700 g/L in MS(-) and 1.760 g/L in MS(+) patients) (*Table II*). MDA shows a higher value in the MS(+) group and is related to ischemic syndromes and more related to inflammation.

Discussion

The metabolic syndrome increases the risk of cardiovascular disease and type 2 diabetes. The growing prevalence of chronic non-communicable diseases is dramatic wordwide and is a global health care problem (19). The National Cholesterol Education Program – Adult Treatment Panel III (NCEP-ATP III) has recognized the metabolic syndrome as a cluster of abnormalities increasing the risk of both cardiovascular disease (CVD) and type 2 diabetes. The EAS guidelines have also underlined the central role of abdominal obesity in the development of this syndrome (14, 20). The main question on the validity and applicability of the definitions is assessed by Laaksonen (21), and how the metabolic syndrome is related to dyslipidemia is described by Blaton et al. (8). Previous studies demonstrated a modest association between C-reactive protein (hsCRP), carotid artery stenosis, and carotid intima-media thickness (IMT) in a general population (22). Therefore, we studied the APP (acute phase proteins) regarding the process of stenosis in cardiovascular patients. In our ongoing study on the inflammation process and

degree of stenosis, hsCRP and fibrinogen are significantly related to the degree of stenosis. In the present study, we followed the influence of the metabolic syndrome on acute phase proteins through the fibrinogen concentration, which was higher in the MS(+) patient group and reached to 3.247 g/L, not significantly higher (P = 0.561) than in the MS(-) patient group with 3.141 g/L. The metabolic syndrome has no direct association with APP. Baseline characteristics of the Asklepios study population with normal persons aged 24 to 65 showed an average ox-LDL concentration of 91.5 ± 38.4 (U/L) for males and 100.7 ± 38.8 (U/L) for females (23). The included patient data show lower values (77 ± 31 (U/L)) for the patient group MS(-) than (88.7 ± 36 (U/L)) for MS(+) patients (P = 0.064). The metabolic syndrome is directly related to ox-LDL and is a major risk factor for cardiovascular events. MDA related to inflammation and to ischemic syndromes and responsible for plaque instability was also mentioned and was increased in the MS(+) group of patients. A very important finding is the highly significant difference in apoB concentrations (P = 0.001) between both groups. ApoB results confirm the observations from the obtained data on low density lipoproteins. The oxidative stress markers are well influenced and significantly changed by the metabolic syndrome. The significant increase in ApoB and an unchanged TC and LDL-c are responsible for increased small LDL particles which are very unstable for oxidation (24, 25). At the same time, the obtained data with apoB underline again the advantage of the apolipoprotein peptide as a better discriminator for earlier detection of the cardiovascular risk (26, 27). The lipid changes in MS(+) patients increased the risk for coronary events and our obtained data confirm ox-LDL and apoB as key laboratory indicators for the prevention and treatment of cardiovascular diseases in patients with the metabolic syndrome.

In conclusion, the important role of inflammation in atherosclerosis, confirmed by prospective epidemiological studies, is demonstrated through increased vascular risk in individuals with elevated levels of a wide range of molecules (1). Increased plasma levels of CRP, SAA, IL 6 and TGF constitute an inflammatory signature of advanced atherosclerosis and correlate with the extent of disease, but do not provide discriminatory diagnostic power (28). In our previous study, we investigated the interrelationship between cytokines and lipid changes in function of the degree of stenosis in cardiovascular patients. The cytokines were inversely related to HDL and especially to Apo (A-I); only IL 10 was directly related. To understand the role of cytokines in cardiovascular events and the influence of the metabolic syndrome on inflammation, we followed the cytokine concentrations in patients with the metabolic syndrome, MS(+), and in patients without MS (MS(-)). We calculated a lightly but significantly higher concentration of IL 10 (P = 0.051) in MS(+) patients. The other analyzed »inflammation« markers were rather unchanged, suggesting the metabolic syndrome has no direct association with the cytokine production. TNF-α and IL 10 are increased in MS(+) patients and the ratio of IL 6/IL 10 is decreased with P = 0.264. The cytokines are more pro-atherogenic in the patient group MS(+). As a general conclusion, the metabolic syndrome has a moderate association with the inflammation response and the major effect is on the reassembly of small LDL particles and on the concentration of oxidized LDL, principal risk factors in the cardiovascular events. ApoB (P = 0.003) emerged as a strong predictor of the ox-LDL concentration, independently of age, gender, BMI and smoking.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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