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Circulating microparticles levels are increased in patients with diabetic kidney disease: A case-control research

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) is associated with chronic lowgrade inflammation. Microparticles (MPs) are extracellular microvesicles released during apoptosis and cellular activation. The MP's pro-coagulant and pro-inflammatory activities are involved in endothelial dysfunction observed in T2DM patients. This study aimed to evaluate the circulating MPs profile in T2DM patients with diabetic kidney disease (DKD) and correlate it with clinical and laboratorial parameters.

Methods: MPs derived from platelets (PMPs), leukocytes (LMPs), endothelial cells (EMPs), and expressing tissue factor (TFMPs) were measured by flow cytometry, in plasma of 39 DKD patients and 30 non-diabetic controls.

Results: We observed higher PMPs, LMPs, EMPs, and TFMPs (all $p < 0.0001$) levels in case group as compared to controls. For patients with DKD, circulating MPs levels were influenced by gender, but not by obesity status nor by T2DM onset. Fasting glucose and 25-hydroxyvitamin D levels showed correlation with circulating MPs levels in both groups.

Conclusions: These results suggest that type 2 diabetes mellitus patients with DKD presented higher circulating MPs levels - PMPs, LMPs, EMPs, and TFMPs - which correlated with metabolic alterations.

1. Introduction

Diabetes mellitus is a complex and chronic illness whose prevalence has reached epidemic proportions in worldwide, becoming the largest global health emergency of the 21st century [1]. According to the International Diabetes Federation (IDF), there were 415 million of diabetic patients worldwide in 2015 (a prevalence of 8.8%) and this number may reach 642 million (a prevalence of 10.4%) in 2040. Type 2 diabetes mellitus (T2DM) is the most common manifestation; occurring in 87–91% of the patients [1].

The pathogenesis of T2DM and its macro- and microvascular complications have been associated with a subclinical chronic inflammation, metabolic stress, and activation of the immune system [2–4]. The increase of visceral adipose tissue mass (hypertrophy and hyperplasia) is associated with a shift in adipokines/cytokines production, immune cell activation (M1-phenotype polarization in the adipose tissue macrophages and cell Th1-type response), and activation of intracellular inflammatory signaling pathways, such as IKKβ/NF-κB and JNK [4,5]. Extracellular vesicles (30–1000 nm of diameter) may be a chief component of this intricate interface among metabolic alterations, cell signaling and inflammatory responses in DM2.

Microvesicles or microparticles (100–1000 nm of diameter) and exosomes (70–150 nm of diameter) are the most well-known types of extravascular vesicles [6]. Microparticles (MPs) are released during apoptosis and activation of many cell types, such as platelets, leukocytes, erythrocytes, endothelial cells, vascular smooth muscle cells,

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; ADA, American Diabetes Association; BMI, body mass index; CKD, chronic kidney disease; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration ratio; EMPs, endothelial cell-derived microparticles; FITC, fluorescein isothiocyanate; HPLC, high performance liquid chromatography; hs-CRP, high-sensitivity C reactive protein; IDF, International Diabetes Federation; IQR, interquartile range; LMPs, leukocyte-derived microparticles; MMPs, monocyte-derived microparticles; MPs, microparticles; PBS, phosphate buffered saline; PMPs, platelet-derived microparticles; SD, standard deviation; SPSS, Statistical Package of the Social Sciences; T2DM, type 2 diabetes mellitus; TF, tissue factor; TFMPs, microparticles that express tissue factor

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under influence of cytokines, thrombin, endotoxins or physical stimuli, as well as shear stress or hypoxia [7]. MPs represent a potentially important strategy of cell-cell communication and they have been associated with cellular activation and apoptosis, since they may carry cell surface proteins (receptors, transporters, antigens) and cell components (cyto/nuclear proteins - annexins, cytokines, growth factors, angiogenic and heat shock proteins; DNA; mRNA; and miRNA) from their cells of origin [8–11]. Although the precise molecular mechanism of MPs formation is unknown, cytoskeletal reorganization and alterations in phospholipid symmetry are essential to their formation [9]. Briefly, after cellular activation or apoptotic stimulus, the membrane remodeling process starts with the formation of blebs. This step requires an increase in intracellular calcium concentration, changes in membrane lipid asymmetry, and cytoskeleton protein reorganization. The loss of plasma membrane asymmetry leads to exposure of phosphatidylserine on the outer leaflet and the release of MPs from the cells [10,12,13].

MPs circulate in plasma of healthy individuals, but they are found in others body fluids, including saliva, urine, bile, tear and synovial and cerebrospinal fluids, although how MPs clearance occurs is still unclear. It has been proposed that clearance of MPs may occur via direct phagocytosis, after opsonization, by endocytosis or by phospholipase-mediated degradation [14].

MPs present pro-coagulant activity by binding to cells via specific adhesion receptors, thereby stimulating both tissue factor (TF) and cytokines production, which promote coagulation, inflammation and endothelial dysfunction [11]. Thus, higher levels of specific MPs subsets have been associated with several disorders, such as inflammatory and prothrombotic diseases [15], thromboembolic events [16], pre-eclampsia [17], polycystic ovary syndrome [18], autoimmune diseases [19], cancer [20], infectious disease [21], and T2DM [22]. Some studies have demonstrated that MPs are independent predictors for diabetes-related vascular complications [23–25].

Regardless the existence of some reports examining the association of MP levels and T2DM [22], the results are still controversial and the relationship with clinical and laboratorial variables is poorly explored, which may add information for their potential application as a biomarker. In this study, we investigated the association among circulating MPs levels (platelet-, leukocyte-, endothelial cell-derived MPs and MPs that express TF) and clinical and biochemical parameters in a group of T2DM Brazilian patients with DKD.

2. Material and methods

2.1. Subjects

Sixty-nine subjects were selected for this cross-sectional case-control study. The case group included 39 patients with clinical and laboratorial diagnosis of T2DM, according to the criteria established by the American Diabetes Association (ADA) [26], while 30 gender- and body mass index (BMI)-matched non-diabetic individuals were included as the control group. The BMI index was calculated dividing the weight in kilograms (Kg) by the square of height in meters (m^2). The waist circumference (WC) was measured between the lowest ribs and the iliac crest.

T2DM patients were recruited at the Clinic of Endocrinology (Santa Casa Hospital, Minas Gerais, Brazil) from June 2012 to September 2013. Control subjects were recruited from the local community, during the same period. The exclusion criteria included the following parameters: age over 70 years, pregnancy, cancer, autoimmune diseases, and recent history of cardiovascular disease (e.g. heart attack, stroke, and thrombosis in the last five years), and current or recent infections and/or inflammatory processes. Controls presented normal fasting glucose levels (60–99 mg/dL) and they were not using hypoglycemic drugs.

All the T2DM patients selected presented diabetic kidney disease (DKD), that was defined, according to ADA criteria, as albumin excretion rate > 30 mg/24 h at least 2 of 3 specimens collections within a 3- to 6-month, but no other coexisting renal diseases from causes other than diabetes [26]. In agreement with the National Kidney Foundation, the chronic kidney disease (CKD) classification was based on glomerular filtration ratio estimated (eGFR) by Cockcroft-Gault equation: stage 1 (eGFR ≥ 90 mL/min/1.73^2), stage 2 (eGFR 60–89 mL/min/1.73^2), stage 3 (eGFR 30–59 mL/min/1.73^2), stage 4 (eGFR 15–29 mL/min/1.73^2), and stage 5 (eGFR < 15 mL/min/1.73^2 or dialysis) [27,28].

The ethics committees of Federal University of Minas Gerais (Minas Gerais, Brazil) - ETIC 0062.0.203.000-11 - and of Santa Casa Hospital (Minas Gerais, Brazil) - 059/2011 - approved this study, in conformation to the ethical guidelines of the Declaration of Helsinki. All participants provided written informed consent.

2.2. Blood sampling

Venous blood samples were collected from each participant in heparin sodium and anticoagulant-free tubes, allowed to clot for 30 min, and centrifuged at 1100 × *g* for 20 min at 25 °C. The supernatant fractions obtained (plasma and serum) were aliquoted in microtubes and stored at − 80 °C until biochemical analysis.

2.3. Biochemical analysis

The fasting glucose levels in the control group were measured in serum samples after eight hour fasting. The tests were performed using the enzyme-colorimetric method, BTR 811 spectrophotometer (Biotron, Brazil), and Glicose-PP kit (Gold Analisa, Brazil), following the manufacturer's instructions.

The high-sensitivity C reactive protein levels (hs-CRP) and HbA1c were measured in serum samples using the immunoturbidimetric method, following the manufacturer's instructions, in a System Vitros Chemistry 5.1 FS (Ortho Clinical Diagnostics, US). The same system was used to measure the creatinine and urea levels through colorimetric reaction (Creatinine and Urea VITROS Chemistry Products - Ortho Clinical Diagnostics, US). All samples were assayed at the same time.

The 25-hydroxyvitamin D [25(OH)D] levels were measured in heparin sodium plasma samples using reversed-phase high performance liquid chromatography (HPLC) with UV detection (Thermo Finnigan Surveyor, US), according to Hymoller and Jensen [29] modified. The 25(OH)D2 (≥ 98% pure) and 25(OH)D3 (≥ 99% pure) were used as standards and 1,α-hydroxyvitamin D3 (1αOHD3 - ≥ 97% pure) as an internal standard (all standards from Sigma Aldrich®). The samples were protected from the light and were assayed at the same time.

2.4. Microparticles flow cytometry assay

The purification of the circulating MPs was performed according to Campos et al. [21] modified. The plasma samples (collected in heparin sodium) were centrifuged at 14,000 × *g* for 5 min at room temperature, in order to obtain platelet-free plasma. The supernatant was aspirated and diluted 1:3 in citrated phosphate buffered saline (PBS), containing heparin. Then, the samples were centrifuged at 14,000 × *g* for 90 min at 15 °C, the supernatant was removed and the resultant MPs pellet was resuspended in 1 × annexin V binding buffer (BD Pharmigen™, US).

Isolated MPs were gated based on their forward (FSC) and side (SSC) scatter distribution, and compared to the distribution of synthetic 0.7–0.9 μm SPHERO™ Amino Fluorescent Particles (Spherotech Inc., US) (Fig. 1A). The presence of phosphatidylserine residues on the MPs surface was assessed for their positive staining with monoclonal antibodies against annexin V labeled with fluorescein isothiocyanate (FITC) (BD Pharmingen™, US). Cell-specific monoclonal antibodies were used to identify the source of the MPs: CD41-PerCP-Cy5.5 (eBioscience, US) for platelet-derived MPs (PMPs), CD45-APC (BD Pharmingen™, US) for