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Regular Article

Increased serum concentrations of pentosidine are related to presence and severity of coronary artery disease

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ABSTRACT

Background: There are limited data regarding the contribution of advanced glycation end products (AGEs) in the presence of coronary artery disease (CAD). We investigated whether serum pentosidine and Nε-carboxymethyllysine (CML) were related to the presence and the severity of CAD.
Methods: 69 Tunisian patients with CAD (≥50% obstruction in ≥ 1 coronary artery), 32 Tunisian patients without CAD but with potential cardiovascular risk factors and 60 Tunisian control subjects were included in a cross-sectional study. Patients were classified as CAD and non CAD patients according to angiographic results. The severity of CAD was assessed using the Gensini score. Serum pentosidine and CML were measured by LC-MS/MS.
Results: Serum pentosidine and CML concentrations were significantly higher in non-CAD patients *vs* control subjects ($P < 0.001$). Serum pentosidine concentrations were significantly higher in CAD patients *vs* non-CAD patients ($P < 0.001$). A multiple logistic regression analysis demonstrated that pentosidine was independently associated with the presence of CAD (OR = 1.52, 95% CI: 1.12-2.07, $P = 0.007$). The area under curve (AUC) determined by ROC analysis was 0.74 (95% CI: 0.64-0.84, $P < 0.001$) and the optimal cut-off value of pentosidine to predict the presence of CAD was 3.2 μmol/mol Lys, with 64% sensitivity and 78% specificity. Furthermore, in a multivariate stepwise regression analysis, pentosidine was independently correlated with Gensini score (standardized β = 0.46, 95% CI: 0.70-1.99, $P < 0.001$).
Conclusions: High concentrations of pentosidine show the presence and the severity of CAD with high sensitivity.

Introduction

Cardiovascular diseases are a major cause of morbidity and mortality in patients with diabetes mellitus [1]. Conventional risk factors such as hypertension, hyperlipidemia and smoking are important in the development of diabetic cardiovascular complications, but they explain only a part of excess risk. Previous studies have suggested that hyperglycemia is also related to increased cardiovascular risk [2,3]. There is a large body of evidence linking both glucose intolerance and insulin resistance to an increased risk of coronary artery disease (CAD) [4]. Several potential mechanisms by which hyperglycemia may contribute to CAD are formation of advanced glycation end products (AGEs), increased polyol pathway flux, increased hexosamine pathway flux and protein kinase C activation [5–7]. Formation of AGEs is initiated by the reaction of reducing sugars with free amino groups of proteins. In this reaction, a labile Schiff base is produced, followed by its rearrangement into Amadori products and, eventually, after further reaction cascades involving oxidative processes, into a wide range of AGEs which are mostly formed by the modification of lysine and arginine residues [8,9].

AGEs affect tissues and cells in three general ways: 1) cellular functions can be altered when intracellular proteins are modified by AGEs; 2) modifications of extracellular matrix proteins result in abnormal interactions between these proteins and with cells; 3) circulating AGEs can bind to AGE receptors (*e.g.* the receptor for AGE, RAGE, which induces receptor-mediated production of reactive oxygen species and activates transcription nuclear factor-κB), thereby leading to deleterious changes in cellular processes [10,11].

Several studies so far have examined the associations between various plasma AGEs and microvascular and macrovascular complications

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in subjects with or without diabetes, and shown that AGEs, including pentosidine, were associated with the development of retinopathy, nephropathy and CAD [12–15]. Furthermore, AGE tissue contents are related to increased arterial stiffness, which is an independent contributing factor for CAD, by inducing collagen cross-linking in the vessel wall [16]. Besides, AGEs contribute to the development of atherosclerosis by binding to RAGE on endothelial cells [10].

Some studies showed a relationship between CML, pentosidine and CAD [17–19]. To the best of our knowledge, however, no study has evaluated the association between these AGEs and the severity of CAD. Therefore, this study is the first to investigate the relationship between well defined glycation products and the severity of CAD.

Subjects and Methods

Study Population

A total of 60 control subjects recruited among healthy blood donors of the Regional Blood Transfusion Centre in Monastir (Tunisia) with no cardiovascular risk factors and 101 patients who referred to the department of cardiology of Fattouma Bourguiba University Hospital in Monastir (Tunisia) for coronary angiography between January 2009 and December 2010 were included into the cross-sectional study. Demographic parameters, risk factors for atherosclerosis (hypertension, diabetes, dyslipidemia, and smoking) and past medical history were recorded. Hypertension was defined as systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg or treatment with oral anti-hypertension drugs. Dyslipidemia was diagnosis to guideline of the National Cholesterol Education Program and/or lipid lowering treatment. Diabetes type 2 was diagnosis according to the American Diabetes Association criteria [20]. All patients admitted to our study had a history of stable angina defined by the presence of chest pain or unstable angina and found to have coronary disease at angiography. Patients with acute myocardial infarctus (AMI) were included in the study. Exclusion criteria were: history of coronary artery bypass graft or angioplasty, renal failure, any acute inflammatory conditions that included infections, trauma or fever. After obtaining informed consent, a questionnaire that contained demographic characteristics including age, gender, cardiovascular risk factors and drug history was completed. The local ethics committee approved the study and informed consent was obtained from all subjects and patients.

Coronary Angiography

Coronary angiography was performed by Judkin's technique [21]. Two experienced cardiologists analyzed the angiographic results. Angiography results were divided into CAD group (≥50% obstruction in ≥ 1 coronary artery) and non-CAD group. The severity of coronary atherosclerosis was evaluated according to Gensini scoring system, which is used to reflect the severity of coronary artery disease (plaque rupture, progression of arteriosclerosis) by calculating the score based on the number of stenotic coronary artery segments, the degree of their lumen stenosis and the localization of stenotic changes and scores it as 1 for 1-25% narrowing, 2 for 26-50% narrowing, 4 for 51-75%, 8 for 76-90%, 16 for 91-99% and 32 for a completely occluded artery. This score was multiplied by a factor according to the importance of the coronary artery [21]. Three groups were drawn up according to the CAD severity score: group 1 (n = 32): namely normal coronary arteries (Gensini score = 0) as non-CAD patients but with high risk cardiovascular factors, group 2 (n = 39): mild CAD (Gensini score ≤ 20) and group 3 (n = 30): severe CAD (Gensini score >20) as CAD patients.

Biochemical Investigations

Blood samples were collected after overnight fasting before coronary angiography. Serum was stored at -80 °C prior to analysis if not immediately processed. HbA_{1c} was assayed using G7 HPLC Analyser (Tosoh Europe N.V.). Serum creatinine, fasting serum glucose, total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C) and triglyceride (TG) concentrations were measured using enzymatic methods by CX9 Auto-chemical analysis instrument (Beckman CX9, USA). High-sensitive C-reactive protein (hs-CRP) and fibrinogen were quantified according to the instructions of the manufacturers using particles enhanced immunonephelometric method (BNII, Dade Behring).

AGE Assays

Sample preparation: total (free and protein bound) serum pentosidine and CML concentrations were measured using LC/MS/MS. Briefly, 35 μl of serum spiked with internal standards (d_2-CML and d_3-MG-H1 used as internal standard for pentosidine) were submitted to acid hydrolysis (6 M HCl, 110 °C, 18 h, final volume: 1 mL). For CML quantification, hydrolysates were evaporated to dryness under nitrogen stream. Dried hydrolyzates were then resuspended in 100 μl of 125 mM ammonium formate, and filtered using Uptidisc PTFE filters (4 mm, 0.45 μm, Interchim, France) prior to LC-MS/MS analysis. For pentosidine quantification, pH of hydrolysates was adjusted to 1.5 prior to a solid phase extraction pre-purification step using Strata X-C column (Phenomenex, France). After elution from SPE column using 5% (v/v) ammoniac in methanol, the samples were dried under nitrogen, resuspended in formate ammonium and filtered as described above.

LC-MS/MS assay: CML quantification was performed using a Kinetex HILIC column (100 × 4.6 mm, 2.6 μm - Phenomenex) with a gradient program composed of 5 mM ammonium formate (pH 2.9) as mobile phase A and 100% acetonitrile as mobile phase B. Detection was performed using an API4000 system (ABSciex, France) in positive-ion mode with an electrospray ionization (ESI) source. Multiple reaction monitoring (MRM) transitions used for quantification were as follows: 205.1 > 130.1 for CML and 207.1 > 84.1 for d_2-CML. Calibration curves have been performed by preparing diluted serum solutions spiked with increased amounts of CML (ranging from 2.5 μM to 80 μM), which have been submitted to the same preanalytical treatments as patient samples.

For pentosidine, chromatographic separation was performed using a Kinetex PFP column (100 × 4.6 mm, 2.6 μm - Phenomenex) with a gradient program composed of 5 mM ammonium formate (pH 2.9) as mobile phase A and 100% acetonitrile as mobile phase B. Detection was performed using an API4000 system in positive-ion mode with an electrospray ionization (ESI) source. Multiple reaction monitoring (MRM) transitions were as follows: 379.2 > 135.1 for pentosidine and 232.2 > 169.1 for d_3-MG-H1. Calibration curves have been performed by preparing diluted serum solutions spiked with increased amounts of pentosidine (ranging from 20 nM to 1.6 μM), which have been submitted to the same preanalytical treatments as patient samples.

In addition, lysine content in hydrolysates was quantified by LC-MS/MS in order to normalize the expression of results. The interassay coefficients of variation were 11% for pentosidine and 8% for CML.

Statistical Analyses

All analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, Illinois, USA). Continuous variables were expressed as mean ± SD, or median (interquartile range), while percentages were used to express categorical variables. Variables such as triglyceride, AGEs and Gensini score that were not normally distributed, as determined by the Kolmogorov-Smirnov test, were logarithmically transformed before analysis and expressed as medians with interquartile range. The chi-square test was used to compare the categorical variables. ANOVA test was used to compare the continuous variables between subgroups. An unpaired Student's *t* test was used for normally distributed variable and the Mann-Whitney *U*-test was used for skewed variables. Spearman