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Original Research Article

N-carboxymethyllysine as a biomarker for coronary artery disease and age-related macular degeneration

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ABSTRACT

Background and objective: An association between coronary artery disease (CAD) and age-related macular degeneration (ARMD) has long been postulated, but exact mechanisms remain unclear. The global prevalence of CAD and ARMD increases and early biomarkers for early diagnosis of these diseases are necessary. The aim of this study was to investigate the plasma level of oxidative stress biomarker CML in patients with and without angiographic findings of atherosclerosis in the coronary arteries (CADath+ and CADath−, respectively) and to assess if there was an association of CAD with ARMD.

Materials and methods: The study enrolled 233 subjects. Based on cardiologic and ophthalmologic examinations, the patients were divided into four subgroups: CADath+ARMD+, CADath+ARMD−, CADath−ARMD+, and CADath−ARMD−. The enzyme-linked immunosorbent assay was used for the measurement of plasma CML levels. Serum lipid levels were determined by an automatic analyzer using conventional enzymatic methods.

Results: CADath+ patients had higher CML concentration compared to CADath− subjects (1.04 ± 0.6 vs. 0.83 ± 0.4 ng/mL, $P < 0.001$). The highest mean CML level (1.12 ± 0.7 ng/mL) was found in CADath+ARMD+ patients. The mean plasma CML concentration was higher in subjects with any of the analyzed diseases compared to CADath−ARMD− subjects. A significant positive association of CADath+ (OR = 2.50, 95% CI 1.60–3.90, $P = 0.0001$), ARMD (OR = 2.08, 95% CI 1.40–3.11, $P = 0.0001$) and both analyzed diseases (OR = 4.67, 95% CI 2.29–9.53, $P = 0.0001$) with an increased level of plasma CML in a logistic regression model adjusting by age was identified.

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Conclusions: The level of CML, an oxidative stress biomarker, reflects the presence of atherosclerosis in coronary arteries and shows a possible link between ARMD and CADath+ via oxidative status.

1. Introduction

Coronary artery disease (CAD) is the leading cause of death worldwide in both men and women [1], and age-related macular degeneration (ARMD) is known to be the major cause of uncorrectable visual impairment in the elderly population of developed countries [2]. ARMD risk factors are in close connection, correlate with, and often are identical to the risk factors of cardiovascular diseases [3]. There is some evidence that both these diseases may share common pathogenesis: morphological and immunohistological changes in ARMD are similar to those in the arterial intima in cases with atherosclerosis [4]. In recent studies atherosclerosis is defined as local arterial intima inflammation, caused by the interplay between lipid metabolism and oxidative stress [5,6].

N-carboxymethyllysine (CML) – a product of both lipoxidation and glycoxidation [7] – represents a general marker of oxidative stress and long-term damage to proteins [8]. In vivo studies revealed strong intracellular CML staining areas of histiocytic/monocytic infiltration or proliferation, mostly associated with atheroma formation [9]. Given that multivessel flow-limiting obstructions are observed in patients with chronic coronary syndrome, atheroma formation and, consequently, atherosclerotic plaques seem to affect coronary flow [10]. Furthermore, CML accumulation detected locally in the eyes of patients with ARMD [11–13] suggests that similar oxidative stress may also show up in the retina, where it can result in tissue damage, and may lead to irreversible central vision loss. It has been stated that relative ischemia of the outer retina that may be caused by atherosclerosis and atrophy of the choriocapillaris is involved in the development of exudative ARMD [14].

Recent studies [2,4–6,15] aiming at preventing the development of CAD and ARMD are still striving to identify the underlying pathomechanisms of both common disorders. We aimed to investigate the plasma levels of CML in patients with and without angiographic findings of atherosclerosis in the coronary arteries; moreover, an attempt was made to assess the potential association of atherosclerosis in coronary arteries with ARMD.

2. Materials and methods

2.1. Study population

The study was performed with the permission No. BE-2-28 issued by the Kaunas Regional Biomedical Research Ethics Committee. A written informed consent was obtained from all participants prior to their inclusion into the study.

The recent case-control study enrolled 233 patients who underwent a detailed cardiologic examination including diagnostic coronary angiography at the Department of Cardiology, Hospital of the Lithuanian University of Health Sciences (LUHS). The inclusion criterion was age ≥45 years for both sexes. Exclusion criteria of the study included diabetes mellitus, serious systemic diseases (somatic illness like oncological or mental disorders); retinal diseases other than ARMD, ocular trauma in the past; and refusal to participate.

The participants were divided into two groups as follows: the CADath− group included the subjects without angiographic findings of atherosclerosis in the coronary arteries and the CADath+ group, the patients with atherosclerotic lesions in the coronary arteries.

CADath− was defined by the absence of any lesion with a diameter of stenosis less than 50% on quantitative coronary angiography of the baseline coronary angiogram. CADath+ was defined as stenosis greater than or equal to 50% of the vessel lumen in one, two, or three main arteries.

All the participants were examined by an ophthalmologist at the Department of Ophthalmology of the LUHS to confirm or rule out ARMD. The ophthalmological examination included best-corrected visual acuity recording using manifest refraction, and the LogMar visual acuity chart, Schiøtz tonometry, slit lamp-assisted biomicroscopy of the anterior and the posterior segments of the eye, and stereoscopic fundus photographs (Carl Zeiss Meditec AG, Germany). Optical coherence tomography (Zeiss Stratus OCT 3000) and fluorescence angiography were performed in patients with suspected exudative ARMD. The diagnosis of ARMD was made if confirmed by two ophthalmologists, and if no other retinal disorders were found during a detailed ophthalmological examination. Finally, the patients were divided into four subgroups: CADath+ARMD+ ($n = 77$), CADath+ARMD− ($n = 67$), CADath−ARMD+ ($n = 47$), and CADath−ARMD− ($n = 42$).

The eye examination was performed and blood samples were taken 3–6 months after coronary angiography. The subjects were asked to abstain from food and drinks for 12–14 h before blood sampling. Total serum cholesterol (TChol), low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides (TG), and plasma CML concentration were measured.

2.2. Laboratory analyses and anthropometric measurements

Blood samples for measurement of CML were collected in vacuum tubes using EDTA as an anticoagulant (EDTA-K3). Plasma samples were prepared within 50–70 min and then stored at −70 °C until analysis. Frozen plasma samples storage time averaged 60 days and thawed only once.

The CML levels in the plasma of the participants (quantity of CML adducts in protein samples) were measured by the enzyme-linked immunosorbent assay (OxiSelect™Nε-carboxymethyl lysine (CML) ELISA Kit, Cell Biolabs, Inc., San Diego, USA). CML-BSA standards and protein samples (100 μL), diluted in PBS (phosphate buffer saline, pH 7.4), were added to the wells of 96-well Protein Binding Plate. The Protein Binding Plate was incubated at the temperature of 37 °C for 2 h. The CML protein adducts present in the sample or the standard were probed with an anti-CML antibody, followed by an HRP-conjugated secondary antibody. Color was developed after incubation with the Substrate solution. After cessation of the reaction followed by adding the Stop Solution to each well, the absorbance was read immediately using an ELISA reader (BIO-TEK ELx800, Bio-Tek Instruments, Winooski, Vermont, USA). The CML concentrations in the samples were determined from the CML-BSA standard curve by comparing its absorbance with the standard curve. An 8-point calibration curve generated from CML-BSA standard serial dilutions was used. The concentration of CML was expressed as ng/mL.

Serum TChol, LDL-Chol, HDL-Chol, and TG levels were determined by an automatic analyzer using conventional enzymatic methods in a certified laboratory of the Laboratory Medicine Department of the LUHS Hospital.

The height (without shoes) of the studied participants was measured with an accuracy of one centimeter, using a statiometer. The body weight of the participants wearing light indoor clothing and no shoes was measured with an accuracy of 0.1 kg, using standardized medical scales. The body mass index (BMI) was calculated as body mass/height^2 (kg/m^2).

2.3. *Statistical analysis*

Statistical analysis was performed by using the IBM SPSS 21.0 software. The following quantitative parameters were calculated: mean (*M*) and standard deviation (SD); the qualitative parameters included the total number (*n*) and percentage (%). To compare mean characteristics between two groups with equal data distribution, Student *t* test was applied. The non-parametric Mann–Whitney *U* test was used for the comparison of two independent samples when the assumption of normality was not satisfied. Logistic regression was applied to assess the odds ratios and 95% confidence interval (CI).

3. Results

A total of 144 patients with atherosclerotic changes in their coronary arteries (CADath+ group) and 89 participants without atherosclerotic lesions in the coronary arteries (based on the results of coronary angiography) (CADath− group) were enrolled to the study. Clinical and laboratory characteristics of the study participants are shown in Table 1.

Plasma CML concentration was markedly higher in CADath + patients compared to CADath− subjects (1.04 ± 0.6 vs. 0.83 ± 0.4 ng/mL, $P < 0.001$). The correlation analysis between CML and lipid levels showed no association (data not shown).

After the eye examination, ARMD was confirmed in 52.8% of CADath− participants (47 of the 89 subjects) and in 53.5% of CADath+ patients (77 of the 144 cases) ($P > 0.05$). Only early-mid stage ARMD was found in study participants. According to the ophthalmologic status, two major groups were divided into subgroups: CADath+ARMD+ ($n = 77$), CADath+ARMD− ($n = 6$), CADath−ARMD+ ($n = 4$), and CADath−ARMD− ($n = 42$). The mean plasma CML concentration was significantly higher in patients with any one of the analyzed diseases (CADath+ or ARMD+) compared to the control (CADath−ARMD−) subgroup (Figure). The highest mean CML level was found in patients with both diseases (the CADath+ARMD+ subgroup), and it significantly differed from that in the control (CADath−ARMD−) subjects (1.12 ± 0.7 vs. 0.76 ± 0.4 ng/mL, $P = 0.001$) and the CADath−ARMD+ subgroup (1.12 ± 0.7 vs. 0.90 ± 0.5 ng/mL, $P = 0.016$). There was a trend of a higher CML concentration in

Table 1 – Clinical and laboratory characteristics of the study participants.

Variable	CADath− group N = 89	CADath+ group N = 144
Men, %	50.7	48.3
Age, years, mean (SD)	69.5 (7.9)	69.2 (8.7)
Smoking, *n* (%)	7 (7.9)	18 (12.5)
Arterial hypertension, *n* (%)	75 (84.3)	131 (90.9)
Body mass index, kg/mm^2, mean (SD)	28.7 (4.7)	28.6 (4.8)
TChol, mmol/L, mean (SD)	5.29 (1.3)	4.78 (1.3)*
LDL-Chol, mmol/L, mean (SD)	2.75 (1.0)	2.64 (0.9)
HDL-Chol, mmol/L, mean (SD)	1.55 (0.8)	1.08 (0.3)*
TG, mmol/L, mean (SD)	1.33 (0.9)	1.65 (1.2)*
CML, ng/mL, mean (SD)	0.83 (0.42)	1.04 (0.59)*

CADath− group, patients without atherosclerotic changes in the coronary arteries; CADath+ group, patients with atherosclerotic changes in the coronary arteries; LDL-Chol, low-density lipoprotein cholesterol; HDL-Chol, high density lipoprotein cholesterol; TG, triglyceride.
* Significant difference between groups ($P = 0.001$).

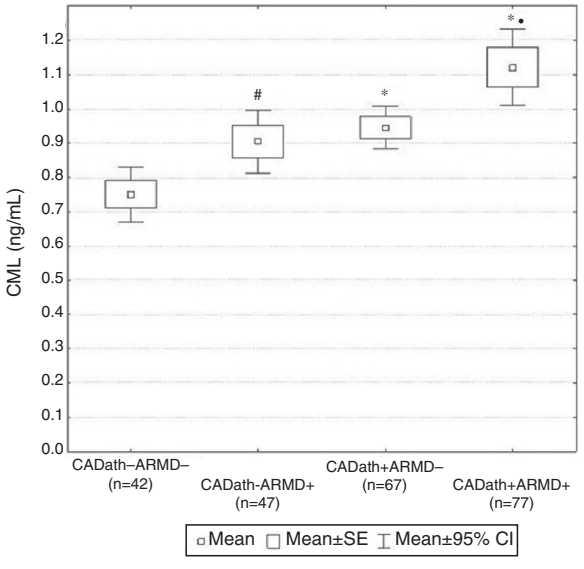

Figure – Plasma levels of N-carboxymethyllysine in the study subgroups. #P < 0.05, compared with CADath−ARMD− group; *P < 0.01, compared with CADath−ARMD− group; •P < 0.02, compared with CADath−ARMD+ group.

Table 2 – Fasting serum lipid concentrations in subgroups of patients without atherosclerotic changes in the coronary arteries.

Lipids profile	CADath−ARMD− N = 42	CADath−ARMD+ N = 47
TChol, mmol/L, mean (SD)	5.14 (1.33)	5.38 (1.29)
LDL-Chol, mmol/L, mean (SD)	2.52 (1.07)	2.89 (1.01)*
HDL-Chol, mmol/L, mean (SD)	1.63 (1.23)	1.51 (1.01)
TG, mmol/L, mean (SD)	1.37 (1.05)	1.29 (0.68)

CADath−, patients without atherosclerotic changes in the coronary arteries; ARMD, age-related macular degeneration; LDL-Chol, low-density lipoprotein cholesterol; HDL-Chol, high-density lipoprotein cholesterol.
* Significant difference between groups ($P = 0.001$).

CADath+ARMD− cases compared to the CADath−ARMD+ subgroup (0.95 ± 0.4 vs. 0.90 ± 0.5 ng/mL, $P = 0.073$), and marginally significant CML concentration difference in the CADath−ARMD+ subgroup compared with the control (CADath−ARMD−) subjects (0.90 ± 0.5 vs. 0.76 ± 0.4 ng/mL, $P = 0.045$).

The majority of CADath+ patients (87.5%) were using lipid-lowering drugs. Thus, the values of fasting serum lipid levels were compared only between the CADath−ARMD+ and the CADath−ARMD− subgroups (Table 2). Patients with ARMD had a significantly higher LDL-Chol concentration than subjects without ARMD (CADath−ARMD+ vs. CADath−ARMD−: 2.89 ± 1.01 vs. 2.52 ± 1.07 mmol/L; $P = 0.001$) did, and a trend of higher TChol levels in the ARMD group was observed (CADath−ARMD+ vs. CADath−ARMD−: 5.38 ± 1.29 vs. 5.14 ± 1.33 mmol/L; $P = 0.065$).

A significant positive association of atherosclerotic CAD (OR = 2.50, 95% CI 1.60–3.90, $P = 0.0001$), ARMD (OR = 2.08, 95% CI 1.40–3.11, $P = 0.0001$) and both analyzed diseases (OR = 4.67, 95% CI 2.29–9.53, $P = 0.0001$) with an increased level of plasma CML in a logistic regression model adjusting by age was identified.

4. Discussion

The present study showed that higher plasma CML levels were associated with atherosclerotic CAD and ARMD. The frequency of ARMD in coronary artery disease patients with or without angiographic findings of atherosclerosis did not differ in our study (52.8% in the CADath+ vs. 53.5% in the CADath− group, respectively, $P > 0.05$). These findings might be due to the study design – the groups of our study (CADath+ and CADath−) were formed of the patients from the Department of Cardiology. As the frequency of ARMD in the general Lithuanian population is known to be 7.3% [16], the higher frequency of ARMD found in our study participants confirmed the statement that the prevalence of ARMD is higher in cardiologic subjects [17,18]. The goal of our research was the investigation of oxidation status relation with atherosclerosis in the coronary arteries and ARMD. Thus, the design of our study was specific and well disposed for such analysis.

Plasma concentration of CML was analyzed by several scientists separately in cardiology patients and ARMD cases [11,12,19–21]. Kralev et al. [19] found significantly elevated CML levels in patients presenting with acute myocardial infarction. The results were explained by the involvement of endothelial dysfunction through CML-receptor interaction and oxidative stress in acute myocardial infarction. Kalousova et al. [20] stated that CML levels were significantly elevated in patients with atherosclerosis compared to healthy subjects. Moreover, the Prospective Cohort Study demonstrated that older adults with high plasma CML were at a greater risk of dying, especially from cardiovascular disease [21]. In agreement with previous studies, our results demonstrated that significantly higher CML levels were observed in patients with atherosclerotic changes in the coronary arteries compared to those without angiographic findings of atherosclerosis (1.12 ± 0.7 ng/mL in the CADath +ARMD+ subgroup vs. 0.76 ± 0.4 ng/mL in the CADath−ARMD− subgroup, respectively, $P = 0.001$).

Blood CML concentration has been measured in a cohort of relatively healthy, community-dwelling adults [22]. Despite that, it is difficult to make a direct comparison of CML levels with other studies because different methodologies were used to measure circulating CML. Semba et al. [22] conducted a study in 26–93-year-old healthy adults, and stated that the serum concentration of CML among the subjects was lower in healthy adults than that described in diabetic subjects. As CML is known to be a product of both lipoxidation and glycoxidation [7], the accumulation of CML seems to be increased in individuals with abnormal glucose metabolism [22,23]. Therefore, we carefully excluded patients with diabetes mellitus from our study.

Given the hypothesis that ARMD is a systemic disease [3] and may share several common pathological mechanisms with CAD [4–6], we also analyzed the association of the CML level with ARMD. To our knowledge, this is the first study to examine the relationship of the CML level with both CAD and ARMD. Our data indicated that a higher plasma CML concentration was found in ARMD cases of both CADath+ and CADath− groups, compared to individuals without ARMD. Ni et al. [11] in their study of 58 ARMD and 32 control subjects found that the mean CML level was significantly elevated in those with early/mid-stage dry ARMD. In the cross-sectional study by Semba et al. [24], where late ARMD were included, no associations of serum CML with early and late ARMD in combination (OR = 0.97; 95% CI 0.90–1.04; $P = 0.44$) or with late ARMD (OR = 0.94; 95% CI 0.82–1.08; $P = 0.36$) were found.

As dyslipidemia is a common risk factor for ARMD and CAD, an analysis of serum lipid levels was performed in our study. We had to exclude the CADath+ group from the analysis because most of those patients (87.5%) were using lipid-lowering drugs. The analysis of serum lipid concentration in patients without angiographic findings of atherosclerosis revealed an atheroprotective phenotype of the lipid profile (lower TChol and LDL-Chol, and higher HDL-Chol concentrations) in the subgroup without ARMD compared to the ARMD subgroup subjects. However, a significant difference between the mean lipid concentration was observed only in the LDL-Chol profile (2.89 ± 1.01 mmol/L in CADath−ARMD+ vs. 2.52 ± 1.07 mmol/L in CADath−ARMD−, respectively). The lipid level had no significant influence on CML in a study conducted by Ahmed et al. [25];

however, the researchers revealed that an increased circulating level of CML was associated with the development of ischemic heart disease. The results of logistic regression in our study identified a significant positive risk of both diseases – atherosclerotic CAD and ARMD – associated with an increased level of circulating CML.

5. Conclusions

The level of plasma CML reflects the presence of atherosclerosis in the coronary arteries. Moreover, our data provide a support for a possible link between early/mid ARMD and atherosclerotic CAD via oxidative pathway: the level of CML, biomarker of oxidative stress, was increased in patients with atherosclerotic CAD or ARMD and it was highest in patients with both diseases. These findings are the background for further research, where the more detailed analysis of systemic CML concentration association with the clinical course of ARMD and CAD would be included.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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