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Carboxymethyl lysine, an advanced glycation end-product, and incident diabetes: a case-cohort analysis of the ARIC Study

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Abstract

Aims—To verify whether elevated fasting levels of circulating carboxymethyl lysine (CML), an advanced glycation end-product (AGE), predict the development of diabetes in middle-age adults.

Methods—Using a stratified case-cohort design, we followed 543 middle-aged individuals who developed diabetes and 514 who did not over a median 9 years in the Atherosclerosis Risk in Communities Study. Weighted Cox proportional hazards analyses were used to account for the design.

Results—In weighted analyses, correlation between CML levels and anthropometric, inflammatory or metabolic variables was minimal (Pearson correlations usually <0.10). CML, when modelled as a continuous variable and after adjustment for age, sex, race, centre, parental history of diabetes, body mass index, waist-to-hip ratio, NEFA, oxidized LDL-cholesterol, glomerular filtration rate, smoking, an inflammation score, adiponectin, leptin, insulin, and glucose levels, was associated with increased risk of diabetes (HR=1.35; 95% CI 1.09 – 1.67, for each 100 ng/mL CML increment). Baseline glucose level and race each modified the association ($p<0.05$ for interaction), which was present only among those with impaired fasting glucose (≥5.6 mmol/l, HR=1.61, 95%CI 1.26 – 2.05) and among whites (HR=1.50, 95%CI 1.13 – 1.99).

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Conflicts of interest

The authors state that they have no conflict of interest.

Conclusions—Elevated fasting CML, after adjustment for multiple risk factors for diabetes, predicts the development of incident diabetes, the association being present among those with impaired fasting glucose and in whites. These prospective findings suggest that AGE might play a role in the development of diabetes.

Keywords

Diabetes; Advanced Glycation End Products; Epidemiology

Introduction

Advanced glycation end-products (AGE) comprise a heterogeneous group of compounds which are formed via a series of non-enzymatic reactions between reducing sugars, proteins and lipids [1]. They influence many biological functions, especially as they are irreversibly deposited over time in various organs and vessel walls [2]. Serum concentrations of AGE have been associated with development of atherosclerosis, microangiopathy, and the severity of diabetes complications, including nephropathy and retinopathy [3,4].

Circulating AGE derive not only from endogenous processes, notably in hyperglycaemic and hyperlipemic states [5], but also from external sources, the latter mainly from foodstuffs. Dry heat, ionization, and irradiation, common industrial processes intended to improve safety, digestibility, transportability, and to enhance flavour of foods, generate AGE. Heat and dehydration employed in home and commercial cooking, especially in broiling, searing, and frying, also significantly increase the content of AGE in foods [5]. In addition, circulating AGE may also derive from tobacco as a result of heat drying in its processing and combustion during its consumption [6].

Animal studies and small clinical experiments in humans aiming to restrict AGE intake from foods, suggest that increased circulating AGE levels may precede, as well as result from, diabetes mellitus [5]. However, prospective evidence from cohort studies of free-living adults concerning the role of AGE in the development of type 2 diabetes, to our knowledge, has not yet been reported. Hence, the aim of the present study is to verify whether elevated fasting levels of carboxymethyl lysine (CML), a circulating AGE, predict the development of diabetes in free living, middle-age adults.

Patients and Methods

The Atherosclerosis Risk in Communities (ARIC) study recruited a population-based cohort of 15,792 individuals aged 45–64 years from four US communities between 1987 and 1989. After a baseline clinic examination (visit 1) ARIC conducted three clinic examinations at approximately 3-year intervals (visits 2–4), and then a fifth visit in 2011–2013, as well as annual telephone interviews. The analyses reported here include information from visits 1–4 and cover an average of 9 years of follow-up [7]. Human-subject research review committees at the involved institutions approved the study, and all participants gave written consent. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

For the present analyses we used a stratified case-cohort design, as previously described in the investigation of the role of several inflammation biomarkers in the development of diabetes in the ARIC Study [7]. Diabetes was defined based on a self-reported physician diagnosis, use of antidiabetic medications, or a fasting glucose value ≥ 7.0 mmol/l at an ARIC visit. Prior to sampling, we excluded 2,018 participants with prevalent diabetes, 95 members of minority ethnic groups with small numbers, 853 individuals who did not return to any follow-up visit, 26 with no valid diabetes determination at follow-ups, 7 with restrictions on stored plasma use, 12 with missing baseline anthropometric measurements, 2,537 participants in previous ARIC case-control and case-cohort studies involving cardiovascular disease for whom stored plasma was either previously exhausted or held in reserve, 45 participants with incomplete fasting (<8 h), and 490 not having values for all covariates in previous ARIC studies regarding diabetes aetiology. This left 9740 eligible individuals. For 31 individuals new markers were not measured. We selected a race-stratified cohort random sample (50% African-American, 50% white), and from the incident cases detected between baseline and the 4th ARIC visit we chose a random sample of diabetes cases, again stratifying by race (50% African-American, 50% white). This resulted in 1095 individuals, for whom sampling weights were calculated. We additionally excluded 38 individuals of the original sample having insufficient remaining plasma to measure AGE. Our final sample was thus constructed from 608 participants selected as the cohort random sample and 449 participants selected as cases. As 94 of those selected in the cohort random sample had developed incident diabetes, our analyses were thus based on a total of 514 non-cases and 543 cases.

Incident diabetes was determined at ARIC follow-up visits, approximately three years apart. The date of onset of diabetes was estimated by interpolation of measured glucose values from the visits immediately prior to and during which diabetes was ascertained, as previously described [7].

We chose to measure CML as a marker of circulating AGE. Despite the fact that the exact chemical structures of most AGE have yet to be determined, several molecular configurations have been structurally identified *in vivo*. CML is among the better characterized AGE compounds, and is frequently used to serve as an AGE marker [5].

We analysed CML at a central laboratory in samples collected at the baseline examination and stored for approximately 20 years at −70°C. CML concentration was measured in duplicate, using an enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of CML (MicroCoat., Germany), according to manufacturer's protocol. This assay does not show significant cross-reactivity with other compounds [8]. The CML ELISA shows a similar response to both free and protein-bound CML and has been validated [9]. Intra- and interassay CV values for CML were 5.5% and 6.5 %, respectively. Although there is a paucity of data on the effects of long-term storage on CML measurements in biological specimens, our quality control (QC) data shows acceptable CML assay performance and is in general agreement with QC data from other studies using the same CML ELISA to determine CML concentrations in specimens stored for extended time periods at −70°C [10,11].

Most covariates were measured at baseline by questionnaire, or physical or laboratory examination. Low-grade systemic inflammation was estimated, as in previous publications from this case-cohort study, by a score ranging from 0 to 6, attributing one point for a value greater than the median of the cohort sample for each of six measured inflammation markers (IL-6, CRP, orosomucoid, sialic acid, white cell count and fibrinogen) [7].

Glomerular filtration rate (GFR), estimated using the CKD-EPI equation, [12] was added as a covariate, as AGE accumulate when renal function is impaired [13].

We used weighted Pearson correlations (and bootstrap confidence intervals) to describe crude associations between CML and other variables, and weighted ANCOVA to compute adjusted CML means in diabetes cases and non-cases. We performed weighted Cox proportional hazards regression to estimate the relation between CML and time to onset of diabetes, with weights defined as the inverse of the race-specific sampling fractions, to account for the design. In proportional hazards analyses, CML was modelled as a continuous variable and as sex-specific quartiles [14]. Adiponectin, body mass index, waist-hip ratio and other continuous variables were centred on their means in order to avoid multicollinearity.

Linearity of the CML association with diabetes was tested according to the Box-Tidwell method [15], considering the Wald test of the variable defined as CML*log(CML), in a model that also contained the original CML variable and all other covariates. In addition to this, the squared term of the centred CML variable was also tested in a similar model. We tested heterogeneity in the CML – incident diabetes association across binary categories of covariates using the Wald test of the interaction coefficient. Statistical analyses were performed using the SAS (SAS Institute Inc., Cary, NC) and SUDAAN (Research Triangle Institute, Raleigh, NC) statistical software packages, reflecting the case-cohort sampling design. The proportional hazards assumption was examined through plots of Martingale and Schoenfeld residuals [16]. Collinearity across independent variables was investigated with linear regression models, variance inflation factors all being < 2.5.

Results

In the weighted cohort random sample, 35.1% of participants were men and 20.2% African-American, 19.2% were obese (body mass index ≥ 30kg/m^2), 21.1% had a parental history of diabetes, 19.7% were current smokers, and 31.9% were former smokers. Median (25^{th} percentile –75^{th} percentile) age was 52 (48–57) years, and median glomerular filtration rate was 68.6 (61.9–76.9) mL/min/1.73m^2. Median CML levels were 246.2 (199.5–291.5) ng/mL. Other characteristics of cases and cohort representative non-cases have been previously reported [7].

Weighted Pearson correlations, assessed in the cohort random sample (n=608), showed no statistically significant crude association between CML and anthropometric (body mass index and waist/hip ratio), and with most inflammatory (C-reactive protein, IL-6, fibrinogen, and orosomucoid), and metabolic (systolic and diastolic blood pressure, adiponectin, leptin, triglycerides, HDL-cholesterol insulin, and fasting glucose) variables. An association of

small magnitude was found between CML and non-esterified fatty acids (NEFA, r=0.30, p<0.001), oxidized LDL-cholesterol (−0.19, p<0.001), and sialic acid (−0.13, p<0.01) (Table 1).

Mean CML values were higher in men than women [269.6 (95%CI 257.1 – 282.1) vs. 241.3 (95%CI 233.1 – 249.4) ng/mL, p<0.001], and also higher, though not statistically significantly so, in African-Americans than in whites [261.3 (95%CI 249.8 – 272.9) vs. 248.7 (95%CI 241.1 – 256.2) ng/mL, p = 0.09], after adjustment for possible confounders (Table 2).

Individuals who developed diabetes during follow up had greater CML values at baseline than those who did not: 263.3ng/mL (95%CI 253.4 – 273.3) *vs.* 250.1 ng/mL (95%CI 243.9 – 256.2) after adjustment for age, sex, race and study centre, parental history of diabetes, body mass index (BMI), waist-to-hip ratio, NEFA, oxidized LDL-cholesterol, renal function, smoking, inflammation score, adiponectin, leptin, insulin and glucose levels at baseline (p=0.03).

Survival analyses for incident diabetes modelling CML as a continuous variable as well as one categorized in quartiles are presented in Table 3. For every 100 ng/ml increment in CML, risk of developing DM increased by 35% [HR = 1.35 (95%CI 1.09 – 1.67)], when adjusting for possible confounders (Model 3). No statistically significant association was found in less adjusted analyses in which CML was modelled as a continuous variable. When CML was modelled categorically, the increased risk [4^{th} vs. 1^{st} quartile HR = 1.44 (95%CI 0.91 – 2.30)] did not meet nominal statistical significance. Squared CML terms were not included in these models, as we were unable to reject linearity of the association between diabetes and CML, either by the Box-Tidwell test (p=0.85) or with the simple addition of a quadratic CML term (p=0.88).

Investigating heterogeneity in the adjusted continuous variable analysis (Model 3), we found no statistically significant effect modification by sex (p=0.14), obesity (p=1.00), current smoking (p=0.79), or below/above median glomerular filtration rate (p=0.46). However, the association differed by baseline glucose level and race. It was present in those with impaired fasting glucose (≥ 5.6 mmol/l; HR=1.61, 95%CI 1.26 – 2.05) but not in those with normoglycemia (HR=0.86, 95%CI 0.55 – 1.35; p for interaction = 0.02). Whites had an approximately 50% increased risk (HR=1.50, 95%CI 1.13 – 1.99), in contrast with African-Americans, for whom no association was found (HR=0.92, 95%CI 0.68 – 1.23; p=0.03 for the interaction).

Discussion

We observed an increased risk of diabetes associated with circulating levels of CML, such that for each 100 ng/ml increment in CML (approximately the CML interquartile range) the risk of developing diabetes increased 35%. The association was present and notably stronger in individuals already presenting impaired fasting glucose at baseline (HR=1.61) and in whites (HR=1.50) than in African-Americans.

Concern that the current epidemics of diabetes and other chronic diseases are related to environmental and societal dynamics that have produced changes in diet and other lifestyle behaviours is widely held [17,18]. How such changes lead to diabetes is a matter of much current investigation. Within this context, increased production and consumption of foods rich in advanced glycation end products, and circulating AGE levels have received growing attention [19]. Serum levels of several AGE molecules are elevated in patients with diabetes, and their levels predict complications and mortality [20,21]. Additionally, in a population-based study of U.S. adults aged 65 and older, higher levels of CML predicted incident cardiovascular events [22], as in older community dwelling women AGE levels predicted cardiovascular disease mortality [23]. However, to our knowledge, the present study is the first large, long-term study to demonstrate a prospective association between circulating AGE levels and incident diabetes.

Though previous animal studies and small clinical investigations provide a rationale for considering AGE as a potentially causative factor, how AGE might cause diabetes is not clear. The literature is quite conflicting with respect to the relationship of AGE to insulin sensitivity. In a study in which 18 patients with type 2 diabetes were randomly assigned to a 4 month period of either a healthy diet or an AGE-restricted diet, the latter achieved by boiling, poaching, stewing, or steaming food, while avoiding frying, baking, or grilling, without changing the quantity or nutrient composition of food [24], a significant decrease in plasma insulin and leptin concentrations and a significant increase in adiponectin levels were observed with the AGE restriction, compared to the control diet, in patients who had diabetes. However, these associations were not significant in the same experiment in 18 healthy individuals [24], despite the fact that serum CML levels were statistically reduced. This results may be expected, as healthy participants started with normal insulin levels and they could not be expected to decrease insulin levels below normal by reducing serum AGEs [24]. In other randomized, crossover, diet-controlled intervention trial with 62 healthy volunteers, HOMA levels were shown to be lower after 1 month of a diet based on mild steam cooking, compared to another that was based on high-temperature cooking [25]. In an observational study, circulating CML levels were associated with insulin resistance (HOMA-IR) in 172 healthy American individuals (an increase of 0.049 ± 0.020 in HOMA-IR for each increase of 1 U/mL in CML, p=0.019) [26], but not in another, where no association was found for CML despite total AGEs were significantly associated with HOMA-IR index, in 322 healthy Japanese participants [27].

In fact, it has been suggested that CML may be more appropriately considered a marker of oxidation rather than of glycation, which could explain a weaker correlation of CML than that of other AGE markers with insulin levels [27]. The low degree of correlation we observed between CML levels and circulating non-esterified fatty acids (NEFA) and oxidized LDL-cholesterol, corroborates this suggestion. It has been proposed that NEFA and CML may share related glycation properties [6], e.g., fatty acids can act as major donors of reactive carbonyls and are believed to be more efficient catalysts of the production of AGE, as well as other advanced lipoxidation end products (ALE), than glucose [3]. Moreover, the inverse correlation found between CML and circulating oxidized LDL-cholesterol levels can be possibly explained by the fact that AGE may contribute to the expression of oxidized LDL receptors in monocyte–derived macrophages, leading to enhanced oxidized LDL

uptake, resulting in foam cell transformation [28]. There was also a small negative crude correlation between sialic acid and CML levels, but we did not find any study in literature that may explain these results. No other significant crude correlation was found between CML levels and clinical variables, including inflammation markers, in the present study. However, we did not measure oxidative stress, suggested as a putative mechanism regarding AGE-incident diabetes association [29]. In a previous study, CML also presented only small negative associations with BMI (−0.15, p<0,001), waist-hip ratio (−0.11, p<0,001) and fasting insulin (−0.09, p<0,001) [22]. In this study we first adjusted analyses for basic confounders (age, sex and race), and then for major diabetes risk factors (obesity and parental history of diabetes), in addition to smoking and renal function (related to AGE formation and clearance), NEFA and oxidized LDL cholesterol (correlated to CML levels in crude analyses), and finally to other well known [30] mediators in the aetiology of diabetes (inflammation score, adipocytokines, and baseline insulin and glucose levels). We presented 3 separated models since controlling for possible mediators may have a different interpretation (looking for a residual association that may occur due to different pathways, not mediated by these known mediators). Unfortunately, we cannot clearly differ confounding from mediation in our data. For instance, increased AGE level can be a marker of higher level of glycaemia, as a confounder, while hyperglycaemia is also an obvious mediator to incident diabetes. The fact that the association between CML and incident diabetes was still significant after controlling for baseline glucose levels thus suggests that other mechanisms may also be involved in this association.

The significant interaction, showing that the association was present only among individuals with impaired fasting glucose might indicate that higher AGE levels may only confer risk during the later phases of the development of diabetes. One could speculate that the combination of external source AGE and AGE produced internally as a result of mild hyperglycaemia overwhelm mechanisms to maintain bodily homeostasis with respect to these molecules. We have no explanation as to why the association we found was present only among white participants.

Our study has several strengths – its relatively large sample of free-living individuals, adjustment for multiple potential confounders, and detailed and repeated ascertainment of incident diabetes. However, its limitations should be acknowledged. We measured only one AGE – CML – and associations with other AGE compounds may be different. We measured CML in long term frozen samples, we have no data on sample stability regarding AGE levels, it was measured only in a fasting state, and we had no measure of AGE content in foods the participants usually consumed. Additionally, the association we found was small, being present only in fully adjusted models, and only when CML was expressed in a continuous form.

In conclusion, this case-cohort study provides evidence, to suggest that elevated circulating AGE levels may increase diabetes risk. Given the supportive findings from animal studies, and the potential for major public health importance of an association of diabetes with this common, modifiable exposure [5], further investigation of this association is warranted.

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What's new?

- This is the first large prospective study of free-living adults concerning the role of an advanced glycation end-product (AGE) in the development of diabetes.
- Our results show increased risk of diabetes associated with circulating levels of carboxymethyl lysine.
- These are relevant findings, since circulating AGE derive not only from endogenous processes, notably in hyperglycaemic and hyperlipemic states, but also from external sources, mainly from foodstuffs, configuring a potentially modifiable exposure.
- The association between CML levels and incident diabetes is especially present among individuals with impaired fasting glucose and in whites.

Table 1

Pearson correlations for crude associations of carboxymethyl lysine (CML) levels with inflammation and metabolic variables at baseline in the weighted cohort random sample. Atherosclerosis Risk in Communities Study.

	Pearson correlation coefficient (95%CI)	
Age (years)	0.017	(−0,078 – 0,116)
N cigarettes per day * N years of smoking	0.029	(−0,063 – 0,128)
Body mass index	−0.058	(−0.147 – 0.029)
Waist	−0.062	(−0.160 – 0.039)
Waist-to-hip ratio	−0.048	(−0.139 – 0.048)
Creatinine	0.031	(−0,055 – 0,114)
Glomerular filtration rate	−0.006	(−0,093 – 0,088)
C-reactive protein	−0.062	(−0,153 – 0,040)
IL-6	−0.012	(−0,085 – 0,058)
Fibrinogen	−0.041	(−0,127 – 0,044)
Orosomucoid	−0.085	(−0,174 – 0,008)
Sialic acid	−0.135	(−0,224 – −0,043) *
White blood count	0.042	(−0,046 – 0,123)
Complement component 3	−0.007	(−0,101 – 0,084)
Adiponectin	0.078	(−0,016 – 0,173)
Leptin	−0.075	(−0,156 – 0,012)
Non-esterified fatty acids	0.301	(0,214 – 0,386) †
Triglycerides	−0.004	(−0,091 – 0,090)
HDL-cholesterol	0.054	(−0,038 – 0,145)
Oxidized LDL-cholesterol	−0.190	(−0,290 – −0,087) †
Systolic blood pressure	0.047	(−0,048 – 0,143)
Diastolic blood pressure	−0.026	(−0,119 – 0,065)
Fasting insulin	0.003	(−0,066 – 0,072)
HOMA-IR	−0.002	(−0,070 – 0,065)
Fasting glucose	−0.074	(−0,160 – 0,013)

* p<0.01;

† p<0.001.

Table 2

Carboxymethyl lysine (CML) levels at baseline in the cohort random sample. Atherosclerosis Risk in Communities Study.

	n	Weighted proportion	Mean CML* (95%CI), ng/mL	*p* value†
Sex				
Women	402	64.9%	241.3 (233.1 – 249.4)	<0.001
Men	206	35.1%	269.6 (257.1 – 282.1)	
Ethnicity				
African-American	295	20.2%	261.3 (249.8 – 272.9)	0.09
White	313	79.8%	248.7 (241.1 – 256.2)	
Age				
45–54 years	409	64.9%	237.9 (218.1 – 257.7)	0.18
55–64 years	199	35.1%	247.7 (240.1 – 255.4)	
GFR (1st quartile) ‡				
Yes	122	24.7%	254.9 (241.2 – 268.6)	0.54
No	486	75.3%	250.0 (243.0 – 257.0)	
Smoking				
Current	133	19.7%	250.3 (237.8 – 262.8)	0.91
Former	183	31,9%	253.6 (240.7 – 266.4)	
Never	292	48.4%	250.0 (241.0 – 259.0)	
Inflammation score				
High	263	37.3%	253.3 (245.2 – 261.5)	0.41
Low	345	62.7%	259.1 (239.2 – 279.0)	
Body mass index				
Normal	194	39.1%	256.3 (245.6 – 267.0)	0.42
Overweight	245	41.7%	246.7 (237.6 – 255.8)	
Obese	169	19.2%	250.6 (235.1 – 266.1)	

*Adjusted for age, sex, race/centre, parental history of diabetes, BMI, BMI^2, waist-to-hip ratio, NEFA, oxidized LDL-cholesterol, glomerular filtration rate (CKD-EPI), smoking (cigarettes/day*years), inflammation score, adiponectin, leptin, ln-insulin, and glucose (baseline values)

†Weighted ANCOVA

‡Glomerular filtration rate < 61.8 mL/min per 1.73 m^2

Table 3

Association of carboxymethyl lysine (CML) with incident diabetes. Atherosclerosis Risk in Communities Study.

	Model 1		Model 2		Model 3	
	HR (95%CI)	**p value**	**HR (95%CI)**	**p value**	**HR (95%CI)**	**p value**
Continuous						
per 100 ng/ml increase in CML	1.09 (0.93 – 1.28)	0.30	1.26 (1.03 – 1.54)	0.06	1.35 (1.09 – 1.67)	0.02
Categorical						
1st quartile	1	0.33 *	1	0.29 *	1	0.30 *
2nd quartile	1.34 (0.96 – 1.87)		1.38 (0.93 – 2.06)		1.28 (0.81 – 2.03)	
3rd quartile	1.08 (0.76 – 1.53)		1.17 (0.78 – 1.75)		1.02 (0.63 – 1.64)	
4th quartile	1.23 (0.88 – 1.73)		1.41 (0.94 – 2.14)		1.44 (0.91 – 2.30)	

Model 1: Adjusted for age, sex, race/centre

Model 2: Model 1 plus parental history of diabetes, BMI, BMI^2, waist-to-hip ratio; NEFA; oxidized LDL-cholesterol; glomerular filtration rate (CKD-EPI) and smoking (cigarettes/day*years)

Model 3: Model 2 plus inflammation score, adiponectin, leptin, ln-insulin, and glucose (baseline values)

*When testing the categorical variable, for an overall association.