

Original

Dietary intake increases serum levels of carboxymethyl-lysine (CML) in diabetic patients

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Abstract

Introduction: Advanced glycation end products are produced endogenously, in association with hyperglycemia and oxidative stress. They can also be generated during cooking or food processing and, once absorbed, alter protein function and promote inflammation.

Methods: We selected 40 healthy male subjects, 17 patients with type 2 diabetes of both sexes and 15 patients with type 1 diabetes of both sexes. Each participant underwent both a food frequency questionnaire (FFQ) and 24-hour dietary recall specially adapted for measuring CML intake, anthropometry, measurement of blood pressure and biochemical parameters in blood and urine.

Results: Serum CML levels were significantly higher in patients with diabetes compared to healthy subjects ($p = 0.04$), showing a direct relationship between dietary intake and serum levels of CML in T2D patients ($r = 0.53$, $p = 0.03$). sCML levels correlated positively with length of diabetes mellitus, and inversely with body mass index (BMI). The most important dietary factor contributing to raise CML levels in these patients with diabetes was the consumption of milk powder.

Conclusion: Serum levels of CML were found to be higher among diabetic subjects, associated to length of diabetes as expected, but also with the ingestion of foods containing higher amounts of ML. The consumption of milk powder in this group is a major determinant of increased serum levels.

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Key words: *Diabetes. Carboxymethyl-lysine (CML). Advanced glycation end products (AGES). Diet. Dietary intake.*

LA INGESTA DIETARIA DE CARBOXIMETIL-LISINA (CML) AUMENTA LOS NIVELES PLASMÁTICOS DE ESTE COMPUESTO EN PACIENTES DIABÉTICOS

Resumen

Introducción: Los productos avanzados de la glicación se producen de forma endógena en relación con la hiperglucemia y el estrés oxidativo. También pueden generarse durante el cocinado o el procesamiento de los alimentos; una vez absorbidos, alteran la función proteica y favorecen la inflamación.

Métodos: Seleccionamos a 40 hombres sanos, 17 pacientes con diabetes tipo 2 de ambos sexos y 15 pacientes con diabetes tipo 1 de ambos sexos. A cada participante se le realizó un cuestionario de frecuencia de consumo de alimentos (CFA) y un recordatorio de 24 horas especialmente adaptado para medir el consumo de CML, antropometría, medición de la presión sanguínea y parámetros bioquímicos en la sangre y la orina.

Resultados: Las concentraciones séricas de CML fueron significativamente mayores en pacientes con diabetes en comparación con los individuos sanos ($p = 0,04$). Se encontró una relación directa entre el consumo dietético y las concentraciones séricas de CML en los pacientes con diabetes tipo 2 ($r = 0,53$; $p = 0,03$). Las concentraciones séricas de CML se correlacionan positivamente con la duración de la diabetes mellitus e inversamente con el índice de masa corporal (IMC). El alimento que más contribuye al aumento de las concentraciones plasmáticas de CML en estos pacientes fue el consumo de leche en polvo.

Conclusión: Se encontró que las concentraciones séricas de CML eran mayores en los sujetos diabéticos, asociado con la duración de la diabetes, como era de esperar, pero también con la ingestión de alimentos que contienen mayores cantidades de CML. El consumo de leche en polvo en este grupo es un factor determinante en el aumento de las concentraciones séricas de CML.

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Palabras clave: *Diabetes. Carboximetil-lisina (CML). Productos de glicación avanzada (AGES). Dieta. Ingesta dietaria.*

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Introduction

AGEs (advanced glycation end-products) are a group of heterogeneous molecules produced by the covalent union of reactive sugars or their oxidation products with proteins, nucleic acids or lipids, through several chemical processes.^{1,2} This occurs through the Maillard reaction and contributes to food organoleptic properties such as color, flavor and aroma, widely employed by the food industry.³

These glycation products can be formed in the body or be incorporated through food or smoking. They are present in the form of peptides immobilized on tissues or free in the extra and intracellular space. Apparently, the intracellular concentration of AGEs is greater than that of plasma.^{4,5} In the presence of hyperglycemia and oxidative stress, AGEs are produced at higher rates.^{1,6} Molecules modified by AGEs circulate throughout the body and exert their action in two main ways: by interacting with receptors or directly by binding covalently to proteins, altering their structure and function. The glomerular damage in patients with diabetes is an example of the latter mechanism.^{7,8}

AGEs are formed in food during cooking with heat by the same reactions as within the body. Several factors influence the formation of AGEs in food, with high temperature being one of the most important. Low moisture, high pH, prolonged cooking time and the presence of some minerals⁹⁻¹² also have an effect, increasing the rate of AGEs formation during cooking. Thus, foods with higher protein or lipid content are more susceptible to the formation of AGEs in the presence of heat and dryness. Several authors have analyzed the content of Carboxymethyl-lysine (ML), one of the most common AGEs, in more than 500 food items.^{4,12-14} Those containing higher levels are of animal origin (meat, cheese) and bakery products.

CML is one of the most studied compounds in the last decade, both in patients with diabetes and in healthy subjects. It is known that about 10% of ingested CML is absorbed from the gut, and 30% of this CML absorbed fraction is excreted by the kidneys.¹³ According to Uribarri et al.¹⁵ plasma CML content increased significantly after an oral load of this compound, showing plasma and urine peaks at 4 to 6 hours post ingestion.

In the U.S. population, the average CML intake is approximately 15,000¹⁶-16,000^{17,18} kU per day and this value is set as the limit for "safe" consumption.¹² It is difficult to establish a number to consider a low dietary intake of AGEs, since it is impossible to reach zero ingestion, but a 60% reduction in CML intake is associated with decreased oxidative stress, less insulin resistance, age related renal function deterioration in humans and improved survival in animals.¹⁹⁻²¹ Animal models fed with isocaloric and isoproteic diets low in CML have shown less visceral fat and weight gain, and lower AGE content in tissues. Animals fed with diets rich in CML, had greater insulin resistance and higher

levels of inflammation.^{19,22} In healthy humans, a high CML diet induces a significant decrease in plasma leptin,²³ increase in pro-inflammatory molecules IL-6, endothelial dysfunction markers as E-selectin, ICAM-1, VCAM-1, TNF α and oxidative stress measured by TBARS or 8-isoprostanes.^{13-16,20,21,23-26} All these effects are probably mediated through the membrane receptor (RAGE).²⁶

Plasma levels of AGEs are usually higher in patients with diabetes than in healthy subjects.^{15,27-29} Chronic endothelial CML accumulation accelerates atherosclerosis and precedes kidney and retinal damage and diffuse coronary artery disease in humans with diabetes and in experimental animals.^{23,30,31} When the diet contains a high amount of AGEs, complications occur earlier and have a faster progression in patients with diabetes.^{15,22,23,27,29,32-35}

The aim of this study was to establish whether a high dietary intake of CML is associated with elevated serum concentration of this compound and increased inflammation (measured as hsCPR) in both patients with diabetes and healthy subjects.

Methodology

Patients

We selected 3 groups of participants. Group 1 included 40 healthy male subjects, aged 25-80 years (31 younger than 50 years, and 9 over 65 years) with the following exclusion criteria: presence of diabetes, chronic renal failure, liver, pulmonary or cardiac failure, invasive cancer or AIDS, smoking (defined as ≥ 5 cigarettes/day for 10 years) and a vegetarian diet. Group 2 was composed by 17 patients with type 2 diabetes (T2D) of both sexes (7 females), aged 50-80 years, and group 3 was composed by 15 patients with type 1 diabetes (T1D) of both sexes (6 females), aged 17-37 years. The last two groups had a diagnosis of diabetes for more than 5 years, and HbA1c less than 9%, and met the same exclusion criteria of group 1 except for the presence of diabetes.

Methods

This study was conducted according to the Helsinki declaration and approved by INTA (Institute of Nutrition and Food Technology), University of Chile ethics committee. After obtaining written informed consent, each participant underwent anthropometry (weight, height, waist and hip circumferences), and blood pressure measurement. After 12 hours fast, blood and urine samples were obtained to measure CML, lipid profile, creatinine, high sensitivity C reactive protein (hsCRP), blood glucose and insulin. Glycated haemoglobin (HbA1c) and microalbuminuria were additionally measured in T1D and T2D. In healthy subjects, serum

glucose and insulin were measured at -30, -15, 0, 15, 30, 60, 90 and 120 min after a 75 g oral glucose load. All measurements, except HbA1c were performed on samples that were frozen at -70° C and analyzed once the study was completed. Routine laboratory parameters (lipid profile, creatinine, glucose, insulin, creatinine, and microalbuminuria) were performed in the Clinical Laboratory VidaIntegra (Santiago, Chile) using automated methods. Hs C-Reactive protein was performed with ELISA kit DRG International Inc. Glomerular filtration rate (GFR) was estimated with Cockcroft-Gault formula.³⁶ HOMA and Matsuda indexes were calculated to assess insulin sensitivity.³⁷

CML intake surveys

Each participant underwent a food-frequency questionnaire (which estimates weekly food consumption)³⁸ and a 24-hour recall (estimates consumption of the day before the assessment day)³⁸ especially adapted to measure CML intake, based on the list published by Uribarri et al.¹² T1D patients also underwent three 24-hour recalls (2 weekdays and 1 weekend day).

CML measurement

Serum CML was measured with a competitive ELISA kit (MicroCoat laboratory Biotechnologie GmbH, Bernried, Germany) where the intensity of color is inversely proportional to the concentration of CML. The inter-assay variation was $0.57 \pm 0.49\%$ (0-4.45%).

Statistical analysis

Data was analyzed with Stata10 for Windows. Variables were assessed for parametric or nonparametric

distribution through Shapiro Wilks test. Descriptive statistics were used to compare mean or median values between groups using analysis of variance or Kruskal Wallis respectively.

The results were expressed as mean and standard deviation if the distribution was normal, or as median and range otherwise. To assess correlations between variables Pearson or Spearman test were used. Multiple linear regression was used to evaluate association between variables.

Results

Demographic and anthropometric data are presented in table I and II.

CML intake survey

Dietary CML intake according to FFQ was 21,945 kU/day (14,767-24,650), 7,314 kU/day (4,129-12,615) and 24,143 kU/day (13,906-27,323) for groups 1, 2 and 3 respectively ($p < 0.001$). Subjects older than fifty years had a significantly lower intake of CML compared to younger subjects both among healthy subjects and patients with diabetes (table IV).

CML intake determined with 24-hour recall was significantly lower compared with FFQ: 8,556 kU/day (5,215-14,277), 3,943 kU/day (2,315-6,106) and 13,640 kU/day (12,094-18,462) respectively in groups 1, 2 and 3. Both surveys (FFQ and 24-hour recall) showed a correlation coefficient of 0.51 in the whole sample ($p < 0.001$). CML intake had a positive and significant correlation with caloric ($r 0.7$), protein ($r 0.6$) and lipid intake ($r 0.76$), both in FFQ and 24-hour recall.

Regression analysis including CML intake by FFQ and consumption of grilled and fried meat, avocados and olives, bread with melted cheese and breakfast cereals; showed that consumption of grilled and fried

Table I
Baseline demographic characteristics of participants by group

Variables	Group 1 healthy subjects	Group 2 T2D patients	Group 3 T1D patients	P
n	40	17	15	
Sex (F/M)	0/40	7/10	6/9	
Age (years)*	46 ^{ab} (42.2-49)	69 ^{ac} (64-70.9)	23 ^{bc} (20-25.8)	abc < 0.001
Actual smoking [†] (yes/no number of patients) (%)	16/24 (40%)	1/16 (6%)	3/12 (20%)	NS
Physical activity [‡] (yes/no number of patients) (%)	10/30 (25%)	0/17 (0%)	5/10 (33%)	NS
Diabetes length (years)*	-	5 (5-6)	14 (7.5-16)	0.001
Hypertension (yes/no number of patients) (%)	8/32 (20%) ^a	15/2 (88%) ^{ab}	1/14 (7%) ^b	0.000
Alcohol intake per day (grams)	17.6 ± 23.1	7.5 ± 15.2	15.8 ± 17.8	NS

*Values expressed as median and confidence intervals.

[†]Less than 5 cigarettes per day.

[‡]Physical activity; yes: more than 3 hours a week.

Table II
Clinical characteristics of participants by age and group category

	Group 1 healthy subjects		Group 2 T2D patients	Group 3 T1D patients	P
N	31	9	17	15	
Age group	<50 years	>65 years	>50 years	<50 years	
BMI*	26.6 (24.6-27.7)	29.2 ^a (25.5-33.6)	29.2 ^b (25.3-33)	23.5 ^{ab} (22.4-24.8)	ab < 0.002
Waist circumference (cm)*	92.5 ^a (88.2-96.7)	95 ^b (92-104)	95 ^c (87-105)	81.5 ^{abc} (77.7-85.8)	abc < 0.001
Waist/hip ratio	d 0.95 ± 0.045 ^d	a 1.01 ± 0.05 ^{ac}	b 0.92 ± 0.08 ^b	ab 0.82 ± 0.04 ^{abcd}	abcde < 0.01
Systolic BP (mmHg)	a 125.8 ± 9.5 ^a	a 146.7 ± 14.4 ^{abc}	c 129 ± 13.4 ^c	b 122.2 ± 12.3 ^b	abc < 0.005
Diastolic BP (mmHg)	80.4 ± 10	76.8 ± 7.4	73.5 ± 8.9	77.1 ± 13.5	NS

*Values expressed as median and confidence intervals.

Table III
Biochemical parameters of participants by age and group category

Variables	Group 1 healthy subjects	Group 2 T2D patients	Group 3 T1D patients	P
n	40	17	15	
Total cholesterol (mmol/l)	4.95 ± 1.1	5.06 ± 0.74	4.45 ± 0.76	NS
HDL cholesterol (mmol/l)	1.22 ± 0.32 ^a	1.1 ± 0.26 ^b	1.57 ± 0.34 ^{ab}	0.002
LDL cholesterol (mmol/l)	2.96 ± 0.96	2.95 ± 0.68	2.5 ± 0.6	NS
Triglycerides (mmol/l)*	1.54 ^a (1.2-1.75)	2.12 ^b (1.52-2.44)	0.7 ^{ab} (0.6-0.9)	0.0001
Fasting plasma glucose level (mmol/l)	5.1 ± 0.46	7.88 ± 1.64	–	0.000
Afterload plasma glucose level (mmol/l)	7.33 ± 2.14	–	–	–
Glycated Haemoglobin (HbA1c) (%)	–	6.98 ± 0.89	7.14 ± 1.02	NS
High Sensitivity C Reactive Protein (mg/dl)*	1.86 (1.40-2.84)	1.93 (1.16-6.16)	0.67 (0.1-3.7)	NS
Plasma creatinine (umol/l)	74.25 ± 10.6	72.48 ± 17.68	69.83 ± 14.14	NS
Glomerular filtration rate (ml/s)	1.97 ± 0.52 ^b	1.58 ± 0.46 ^{ab}	2.3 ± 0.32 ^a	0.02
Urinary Albumin/creatinine ratio (mg/g)*	NS	3.4 (0-12.4)	0 (0-2.9)	NS

*Values expressed as median and confidence intervals.

meat and avocados and olives, significantly determines CML intake in the whole group (p 0.000). In subjects with diabetes (type 1 and 2) the consumption of avocados and olives, bread with melted cheese and grilled and fried meat were also significant in defining total CML intake (p < 0.02). These items determined 80% of total CML ingestion in this group.

Serum CML (sCML)

CML serum levels in the entire sample averaged 653 ± 115 ng/ml (427-933), with no significant differences between sexes. Patients with diabetes had significantly higher levels of CML compared with healthy subjects (684 ± 112 ng/ml and 628 ± 112 ng/ml, p 0.04).

In the whole study group, there was a negative correlation between BMI, waist circumference and sCML

levels (r - 0.3 p 0.009, r -0.33, p 0.04 respectively). No laboratory parameter (table III) was associated with sCML, only GFR was associated specifically with this variable among healthy subjects (r -0.34 p 0.03).

In T2D patients there was a positive relationship between CML intake measured by FFQ and CML serum levels (r 0.53 p 0.03). There was also a positive relation between sCML levels and lipid intake in T1D and T2D subjects (r 0.57, p 0.01).

Milk powder intake was an important contributor to higher sCML levels in patients with T2D (r 0.58 p 0.01) (table V); sCML level was 739 ± 125 ng/ml among those who consumed milk powder compared to 609 ± 81 ng/ml sCML in patients that did not, without differences in HbA1c levels, BMI, basal glycaemia and GFR. In the whole sample, this difference did not reach statistical significance (712 ± 110 ng/ml and 639 ± 113 ng/ml among consumers and non consu-

Table IV
CML intake measured by FFQ and 24 hours recall and sCML by age and group category

	Group 1 healthy subjects		Group 2 T2D patients	Group 3 T1D patients	p
	< 50 years	> 65 years	> 50 years	< 50 years	
Serum CML (ng/ml)	616.8 ± 116.7 ^a	667.3 ± 91.6	655.3 ± 114.8	719.5 ± 102.6 ^a	a < 0.02
CML intake (FFQ)(kU/day)*	22,644 ^{abc} (16,549-28,113)	9720 ^a (7,746-22,301)	7314.5 ^b (4,129-12,615)	24,143 ^c (13,906-27,323)	abc < 0.000
CML intake (24 h recall) (kU/day)*	8,556 ^a (5,215-14,277)	9,223 (3,160-18,716)	3,943 ^c (2,315- 6,107)	13,640 ^c (12,094-18,462)	abc < 0.001

*Values expressed as median and confidence intervals.

mers of milk powder, respectively, p 0.054). Serum CML was also associated with fried and grilled meat intake in subjects over 65 years (r 0.59 p 0.007), consumption of cookies and pastry (r 0.34 p 0.01) and bread with melted cheese (r 0.32 p 0.02) in subjects younger than 65 years.

A negative association between alcohol intake and sCML was suggested by the analysis of extreme values of both variables. Subjects with lowest sCML concentration (10th centile) drank 21.9 ± 18 g/day of alcohol compared to those with highest sCML (90th centile) whose mean alcohol intake was 5 ± 5.6 g/day (p 0.03). In fact, those whose consumption was more than 50 g/day showed lower sCML levels than those subjects with lower than 50 g/day intake (586 ± 119 vs 661 ± 111 ng/ml sCML, p 0.08).

A multiple stepwise regression analysis including the sCML as dependent variable, presence of diabetes, age, CML intake, alcohol and powder milk intake, and BMI, accepted only BMI and presence of diabetes as significant predictors of sCML levels in the whole sample (p < 0.04). Among diabetic participants, where length of diabetes was also incorporated to the model, this last factor and milk powder intake were predictors of sCML (p < 0.03).

Mean serum levels of hsCRP were normal (less than 3 mg/dl) in both healthy and diabetic patients. There was no relationship between hsCRP and sCML in any group (table III), neither with CML intake, BMI, GFR, levels of smoking or physical activity.

Discussion

In this study, we found an association between sCML and CML dietary content in patients with diabetes, in agreement with previous findings in the literature.^{3,15,21,24,39} We also found an inverse relationship between sCML and BMI and alcohol intake.

The inverse relationship between sCML levels and BMI has been previously described, and attributed to CML deposit in fatty tissue, thereby decreasing their circulating levels,⁴⁰⁻⁴² although this is still unclear. It has also been shown that alcohol consumption (acetaldehyde levels) has a protective effect in the formation

Table V
Correlation of specific food items intake with serum CML

	Whole group (72 participants)	Type 2 diabetic patients (17 participants)
	r (p)	r (p)
Milk powder	0.23 (p 0.056)	0.58 (p 0.01)
Mayonaise	-0.26 (p 0.02)	NS
Cookies and bakery products	0.32 (p 0.02)	NS
Light Coke	0.25 (p 0.03)	NS
Regular Coke	NS	-0.51 (p 0.03)
Fresh bread	-0.22 (p 0.06)	NS

of AGEs, by joining to AGEs precursor molecules, preventing progression to advanced glycation end products.⁴³

We found higher CML levels in patients with diabetes, compared to healthy subjects, which could be explained by many factors, including hyperglycemia. Other groups have described levels of 800 to 1.000 ng/ml in patients with type 1 and 2 diabetes, with and without complications.^{44,45} These levels are higher compared to those of our study groups, probably because our patients were metabolically compensated and they did not have microangiopathy (none had microalbuminuria). This fact could also explain the lack of association between sCML levels and GFR in this group. Serum AGEs are cleared by kidneys,^{20,29} in healthy subjects sCML levels return to normal after 18 to 20 hours after oral load of AGEs, whereas in patients with mild diabetic nephropathy this occurs in 36 - 48 hrs and in renal failure patients this occurs after 48 hours.¹³

Dietary CML intake in this sample is similar to that reported in North American populations. Intake level of most participants exceeded the limit of 15,000 kU/day, set as a "safe" limit.^{15,24,46} However, in contrast with reports in the United States,¹⁶ subjects over 65 years consumed significantly less CML. This can be explained by Chilean traditional cooking methods that include mostly boiled rather than fried meats. The

opposite was observed in young subjects in which their cooking methods and food intake is “westernized”, favoring the intake of junk food. From our data, it appears that consumption of specific foods rather than total consumption of CML had the greatest influence on CML serum levels, which can be seen in the significant relationships that sCML show with certain foods, like milk powder, fried and grilled meat, cookies and pastry and bread with melted cheese.

The importance of milk powder intake is noteworthy, mainly among participants with diabetes. However, this could represent a local problem, since government supplies elderly (healthy subjects older than 65 years, and patients with chronic diseases (hypertension, type 2 diabetes) older than 60 years) with a specially formulated dairy powdered drink, which is probably stored for long periods of time. Elevation of AGE levels in products such as milk powder, meat and cheese (with high levels of lysine), occur during high temperature drying⁴⁷ and storage at room temperature.³ Its relevance has been studied in animals, and its importance has also been confirmed in infants, showing that formula fed children have higher sCML^{48,49} which are comparable to adult levels. This has also been shown in teenagers in relation to cocoa powder intake.⁵⁰

The main weakness of this study was the reduced sample size, splitting into different groups according to age and disease, further decreases the sample for different analyses.

It is very important to highlight that there is still no consensus on serum AGE level measurement. Although CML is the most quantified and described, it is not known whether this is the one with greater biological activity, with many compounds described and many also still not been identified.^{50,51}

Our study confirms that consumption of specific food, especially if they are elaborated in dry and high temperature settings, such as milk powder and fried-grilled meat, is essential in increasing serum CML levels, especially among the diabetic population.

Authors contributions

N. J., designed the study, researched data, wrote manuscript, reviewed/edited manuscript. M. J. L., researched data. D. B., reviewed/edited manuscript, contributed to discussion. G. B., researched data, contributed to discussion. L. L., researched data, contributed to discussion. S. H., reviewed/edited manuscript, contributed to discussion. M. P. M., designed the study, obtained support, research data, contributed to discussion reviewed/edited manuscript.

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