Circ J 2017; **81:** 1183–1190
doi:10.1253/circj.CJ-17-0032

ORIGINAL ARTICLE
Preventive Medicine

Chromium Exposure and Risk of Cardiovascular Disease in High Cardiovascular Risk Subjects

— Nested Case-Control Study in the Prevention With Mediterranean Diet (PREDIMED) Study —

Mario Gutiérrez-Bedmar, PhD; Miguel Ángel Martínez-González, MD, PhD; Carlos Muñoz-Bravo, PhD; Miguel Ruiz-Canela, PhD; Alberto Mariscal, PhD; Jordi Salas-Salvadó, MD, PhD; Ramón Estruch, MD, PhD; Dolores Corella, PhD; Fernando Arós, MD, PhD; Monserrat Fito, MD, PhD; José Lapetra, MD, PhD; Lluís Serra-Majem, MD, PhD; Xavier Pintó, MD, PhD; Ángel Alonso-Gómez, MD, PhD; Olga Portoles, PhD; Miquel Fiol, MD, PhD; Mónica Bulló, PhD; Olga Castañer, MD, PhD; Emilio Ros, MD, PhD; Enrique Gómez-Gracia, MD, PhD

Background: Epidemiological data on chromium (Cr) exposure and the risk of cardiovascular disease (CVD) are still limited. Toenail Cr level (TCL) provides a time-integrated measure reflecting long-term Cr exposure. We measured TCL to assess the hypothesis that long-term Cr exposure was inversely associated with incident CVD in a population at high risk for CVD.

Methods and Results: The associations between TCL and CVD were evaluated in a case-control study nested within the "PREvención con DIeta MEDiterránea" (PREDIMED) trial. We randomly selected 147 of the 288 patients diagnosed with CVD during follow-up and matched them on age and sex to 271 controls. Instrumental neutron activation analysis was used to assess TCL. In-person interviews, medical record reviews, and validated questionnaires were used to assess covariates. The fully adjusted OR for the highest vs. lowest quartile of toenail Cr was 0.54 (95% CI: 0.26–1.14; P_{trend}=0.189) for the nested case-control study. On stratification for diabetes mellitus (DM), OR was 1.37 (95% CI: 0.54–3.46; P_{trend}=0.364) for the DM group, and 0.25 (95% CI: 0.08–0.80; P_{trend}=0.030) for the non-DM group (P for interaction=0.078).

Conclusions: The present findings, although not statistically significant, are consistent with previously reported inverse associations between TCL and CVD. These results, especially for non-DM patients, increase the limited epidemiological knowledge about the possible protective role of Cr against CVD. (Trial registration: www.controlled-trials.com; ISRCTN35739639.)

Key Words: Cardiovascular disease; Chromium; Diabetes; PREDIMED; Toenail biomarker

Received January 15, 2017; revised manuscript received February 19, 2017; accepted March 2, 2017; released online April 8, 2017 Time for primary review: 18 days

Department of Preventive Medicine and Public Health, University of Málaga, Málaga (M.G.-B., C.M.-B., A.M., E.G.-G.); The PREDIMED (Prevención con Dieta Mediterránea) Research Network (RD 06/0045), Instituto de Salud Carlos III (ISCIII), Madrid (M.G.-B., M.Á.M.-G., M.R.-C., J.S.-S., F.A., L.S.-M., X.P., Á.A.-G., M.B., E.G.-G.); Department of Preventive Medicine and Public Health, University of Navarra Medical School, Pamplona, Navarra (M.Á.M.-G., M.R.-C.); CIBER Pathophysiology of Obesity and Nutrition, Carlos III Health Institute, Madrid (M.Á.M-G., M.R.-C., J.S.-S., R.E., D.C., F.A., M. Fito, J.L., L.S.-M., X.P., Á.A.-G., O.P., M. Fiol, M.B., O.C., E.R.), Spain; Department of Nutrition, Harvard TH Chan School of Public Health, Boston, MA (M.Á.M.-G.), USA; Human Nutrition Unit, Biochemistry and Biotechnology Department, IISPV, Rovira i Virgili University, Reus (J.S.-S., M.B.); Department of Preventive Medicine and Public Health, School of Medicine, University of Valencia, Valencia (D.C., O.P.); Department of Cardiology, University Hospital of Araba, Vitoria (F.A., Á.A.-G.); Department of Family Medicine, Research Unit, Sanitary District Primary Care Sevilla, Sevilla (J.L.); Department of Clinical Sciences, University of Las Palmas de Gran Canaria, Las Palmas de Gran Canaria (L.S.-M.); Department of Internal Medicine, Hospital Belvitge, Barcelona (X.P.); Palma Institute of Health Research (IdISPa), Hospital Son Espases, Palma de Mallorca (M. Fiol); Cardiovascular Risk and Nutrition Unit, Hospital del Mar Institut for Medical Research (IMIM), Barcelona (M. Fito, O.C.); and Lipid Clinic, Endocrinology and Nutrition Service (E.R.), Department of Internal Medicine (R.E.), Hospital Clinic, Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Barcelona, Spain

Mailing address: Mario Gutiérrez-Bedmar, PhD, Dpto. Salud Pública y Psiquiatría, Facultad de Medicina. Universidad de Málaga, Boulevard Louis Pasteur, 32, 29071 Málaga, España. E-mail: bedmar@uma.es

ISSN-1346-9843

Chromium (Cr) is one of the most common elements in the Earth's crust and seawater, and is found in the environment primarily in 2 valence states, trivalent (+3) Cr and hexavalent (+6) Cr. Trivalent Cr is an essential trace element present in most foods and has very low toxicity. Hexavalent Cr is much less abundant, highly toxic for humans, and largely synthesized by the oxidation of trivalent Cr. The nutritional importance of trivalent Cr started in the 1950s when the need for Cr to maintain normal glucose tolerance in rats was reported.[1] In 1977 the essentiality of Cr for humans was established, when a patient who received total parenteral nutrition developed glucose intolerance, insulin resistance, weight loss and peripheral neuropathy. This situation was resolved with i.v. trivalent Cr.[2] Through the next decades, the requirement of Cr for normal carbohydrate, lipid and protein metabolism was established,[3–6] but the essentiality of Cr has been questioned in recent years due to the failure to identify the underlying biological mechanisms of its action.[7,8]

Despite the fact that more than 40 years ago Schroeder hypothesized that Cr deficiency represented a significant risk factor for cardiovascular disease (CVD),[9] epidemiological data on Cr intake and the risk of CVD are still limited. One limitation is the use of serum or urinary Cr measurement, which may not adequately reflect long-term exposure. Several studies have used toenail Cr level (TCL) as a measure of long-term Cr exposure,[10–12] and 2 of them assessed cardiovascular endpoints.[10,12] These 2 studies were conducted in men only, therefore the findings cannot be generalized to women, but all the aforementioned studies suggested that Cr might play a protective role against CVD.

The purpose of this study was therefore to assess the hypothesis that long-term Cr exposure is inversely associated with the risk of CVD in a population of Spanish adults aged 55–80 years, at high risk for CVD. In order to do this, we developed a nested case-control study within the "PREvención con DIeta MEDiterránea" (PREDIMED) study.

Methods

Study Design

The design and methods of the PREDIMED trial have been described previously.[13,14] The PREDIMED trial was a randomized, controlled, CVD prevention trial based in 11 centers throughout Spain.[15] Institutional review boards at all participating centers approved the study protocol. The study began in October 2003 and was stopped because of early benefit by 1 December 2010.

Eligible participants included men (55–80 years) and women (60–80 years) at high risk for developing CVD at enrollment, but who had never been diagnosed with CVD. High risk was defined as having type 2 diabetes mellitus (DM) or at least 3 of the following major risk factors: current smoking, hypertension, elevated low-density lipoprotein cholesterol (LDL-C), overweight or obesity, or a family history of premature coronary artery disease (CAD). A total of 7,447 participants were recruited between 2003 and 2009. After providing written informed consent, they were randomized to either a traditional Mediterranean diet supplemented with either extra virgin olive oil or tree nuts, or a control (low-fat) diet. The primary CVD endpoint was defined as non-fatal acute myocardial infarction (AMI), non-fatal stroke, or cardiovascular death.

Subject Selection

Among the 7,447 PREDIMED participants, 7,232 (97.1%) provided toenail clippings at baseline, within 6 months of randomization. Of these, we randomly selected 147 of the 288 patients with incident CVD. Median follow-up period from time of toenail sampling to time of incident CVD was 4.8 years (IQR, 3.0–5.8 years). Outcomes were identified through repeated contact with participants, contact with family physicians, annual medical record review, and consultation of the National Death Index.

Patients were randomly matched on age (within 2 years) and sex to 271 controls who were free from CVD before December 2010 and had provided toenail samples at baseline. Most CVD patients were matched to 2 controls, but 23 were matched to only 1 control.

Measurement of Exposure

Toenails incorporate elements as they grow. Once the nail is formed (and expelled from the nail bed), it is then isolated from the metabolic activities of the body. The toenail reflects body intake or exposure from a few months to 1 year.[16] Toenail clippings were stored in small plastic bags at room temperature, and were washed according to International Atomic Energy Agency guidelines. TCL in the stored toenails were measured using instrumental neutron activation analysis (INAA) at the Interfaculty Reactor Institute at Delft University of Technology in Delft, Netherlands. In each series of samples, a blank capsule was analyzed along with the rest to safeguard against Cr contamination in the analysis process. A precise description of the analytical methodology has been published elsewhere.[17,18]

Cr was detected in all samples. The detection limit for a sample of average weight (65 mg) was 0.12 μg/g, and it varied between 0.04 μg/g (sample mass, 250 mg) and 6.56 μg/g (sample mass, 0.53 mg).

TCL INAA measurement is reproducible to 0.1% from date to date. In each series of samples, a reference material was incorporated (INCT-PVTL-6). This material has only a recommended value for Cr, but the INAA laboratory monitor their neutron spectrum with a flux monitor containing Cr that is directly traceable to the NIST standard solution with which the flux monitors are prepared: SRM 3112a – Cr standard solution. Each year, all results are reviewed from all reference materials and the bias for Cr as emerging from that review is 1.006±0.011 (1 SD) across 9 reference materials covering certified values from 0.05 to 200 mg/kg. During the year, 400 of these analyses are repeated.

Covariate Assessment

All the covariates were measured at baseline and yearly during follow-up. We reviewed medical records and used standardized validated protocols[19] to collect information on sociodemographics, lifestyle, health, family history, medication use and medical diagnosis. We grouped medications used habitually by participants at baseline into 8 categories: angiotensin-converting enzyme inhibitors; diuretics; statins; insulin; aspirin-antiplatelet drugs; calcium channel blockers; angiotensin II receptor antagonists; and β-blockers. A validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire[20] was used to evaluate physical activity. Trained nurses measured

Table 1. Baseline PREDIMED Subject Characteristics

Characteristics	Case participants (n=147)	Control participants (n=271)	P value
Age (years)	70 (64–75)	69 (64–74)	Matching factor
Women	40.3	40.5	Matching factor
Toenail Cr (μg/g)† (geometric mean)	0.61	0.72	0.215
Toenail methylmercury (μg/g)†	0.48	0.55	0.073
PREDIMED trial arm			0.031
Mediterranean diet+EVOO	37.4	37.6	0.964
Mediterranean diet+nuts	24.5	35.1	0.026
Current smoker	19.7	15.1	0.229
Hypercholesterolemia	57.1	65.3	0.100
Hypertension	80.3	77.5	0.509
Type 2 DM	61.2	49.1	0.017
Family history of CAD	19.7	15.5	0.271
BMI (kg/m^2)	29.7±3.6	29.7±3.5	0.937
Physical activity (METs-min/day)	250.2±211.1	279.0±272.5	0.267
Alcohol (g/day)	10.1±16.8	12.7±17.9	0.150
Dietary intake			
Total energy intake (kcal/day)	2340±645	2363±599	0.715
Total fat (%E)	39.5±6.5	39.7±6.6	0.700
Monounsaturated fat (%E)	19.6±4.4	20.0±4.6	0.468
Polyunsaturated fat (%E)	6.2±2.2	6.2±1.8	0.923
Saturated fat (%E)	10.4±2.3	10.1±2.2	0.285
Carbohydrates (%E)	41.4±7.3	41.0±7.0	0.615
Cholesterol (mg/day)	363.0±122.2	366.5±130.9	0.784
Fiber (g/day)	24.9±10.3	25.1±7.9	0.784
Mediterranean diet adherence (0–14)	8.2±2.0	8.8±1.8	0.001

Data given as median (IQR), %, mean±SD or †geometric mean. %E, percentage of total energy intake; BMI, body mass index; CAD, coronary artery disease; Cr, chromium; DM, diabetes mellitus; EVOO, extra-virgin olive oil; MET, metabolic equivalent; PREDIMED, PREvention with MEDiterranean DIet.

weight and height using standardized procedures, and blood pressure using a validated semiautomatic oscillometer in triplicate (Omron HEM_705CP). We developed and validated a 14-item Mediterranean Diet adherence tool[21] to assess adherence. We also used a full-length validated food frequency questionnaire[23] to calculate dietary intake. Primary care doctors assessed participants for hypercholesterolemia, hypertension and type 2 DM diagnoses. The concentration of total methylmercury in the stored toenails was also assessed using INAA at the Interfaculty Reactor Institute at Delft University of Technology in Delft, Netherlands.

Statistical Analysis

Data are given as mean±SD or as percentages for continuous and categorical variables, respectively. Group comparisons were carried out using t-test or chi-squared test as appropriate. TCL (μg/g) was categorized into quartiles based on the distribution among controls.

Adjusted levels of covariates across quartiles of Cr in controls were estimated using ANOVA. Whether these adjusted levels were associated with quartiles of Cr was evaluated with polynomial contrast (linear or quadratic trend).

To examine the association of TCL with CVD in the nested case-control study, we used multivariable-adjusted conditional logistic regression, matched for age and sex. We investigated possible interactions between TCL and potential effect modifiers (age, sex and DM) by adding a corresponding multiplicative interaction term in the models, followed by the likelihood ratio test.

Due to the high proportion of DM patients at baseline (53.4%), and the association of Cr with insulin action and DM,[6] we studied the association of TCL with CVD separately for the DM and non-DM patients. For these separate analyses, we used multivariable-adjusted unconditional logistic regression, adjusting for matching factors. For each subgroup, the distribution of Cr in controls was used to calculate cut-off points for quartiles of exposure.

Given that the outcome variable (CVD) consisted of AMI, stroke and cardiovascular death, we carried out a subgroup analysis of outcome for MI and stroke.

We estimated OR and 95% CI using the lowest quartile as the reference category. Tests for trend were performed by assigning each subject the median value of the quartile of Cr and treating it as a continuous variable. We also used this variable to create the multiplicative interaction terms already mentioned. To control for potential confounders, we included in all models those variables based on clinical relevance and previous causal knowledge. We adjusted for the following factors: sex, age, center, smoking, hypertension, hypercholesterolemia, DM, family history of premature heart disease, body mass index (BMI), alcohol intake, sample mass, intervention group, baseline adherence to Mediterranean diet, physical activity, total energy intake, toenail methylmercury level, diuretic use and insulin use.

All P-values reported are 2-tailed, and $P<0.05$ was considered statistically significant. All statistical analysis was

Table 2. Healthy Controls: Adjusted† Baseline Characteristics vs. TCL Quartile (n=271)

Variables	TCL quartiles‡				
	Q1	Q2	Q3	Q4	P for linear trend
No. participants	68	68	68	67	
Median Cr (μg/g)	0.18	0.45	1.09	2.90	NA
Age (years)§	70.08	68.19	68.81	67.39	0.024
Female sex¶	25.69	39.32	38.00	58.14	<0.001
Toenail mass (mg)	54.74	62.08	72.70	70.15	0.050
BMI (kg/m^2)	30.12	29.06	29.25	30.34	0.011††
Primary education or less	85.33	76.12	76.85	80.53	0.523
PREDIMED trial arm					
Mediterranean diet+EVOO	40.33	37.97	38.24	33.97	0.495
Mediterranean diet+nuts	30.99	30.46	33.60	45.33	0.084
Smoke status					
Current	10.85	18.04	15.89	15.75	0.511
Former	34.79	37.84	26.99	31.73	0.398
Hypercholesterolemia	70.62	66.12	60.56	63.95	0.341
Hypertension	79.69	72.17	78.16	79.98	0.770
Type 2 DM	61.79	48.98	40.78	44.70	0.031
Family history of CAD	11.19	20.52	19.20	11.02	0.049††
Physical activity (METs-min/day)	277.34	297.67	292.44	247.90	0.528
Alcohol (g/day)	13.68	13.01	11.93	12.07	0.518
Glucose (mg/dL)	134.16	120.76	117.27	127.38	0.419
Triglycerides (mg/dL)	129.83	121.46	138.34	125.88	0.897
Total cholesterol (mg/dL)	205.84	208.84	212.36	207.29	0.749
HDL-C (mg/dL)	53.81	55.76	50.65	55.50	0.997
Mediterranean diet adherence (0–14)	8.92	8.92	8.95	8.56	0.296
Dietary intake					
Total energy intake (kcal/day)	2,330.24	2,366.56	2,396.35	2,359.73	0.710
Total fat (%E)	40.28	40.01	38.93	39.67	0.408
Monounsaturated fat (%E)	20.46	20.12	19.65	19.62	0.208
Polyunsaturated fat (%E)	6.03	6.26	6.09	6.48	0.230
Saturated fat (%E)	10.22	10.31	9.79	10.24	0.710
Carbohydrates (%E)	39.84	41.04	42.08	41.17	0.192
Cholesterol (mg/day)	368.88	366.92	357.45	372.98	0.969
Fiber (g/day)	23.85	24.94	27.30	24.41	0.348
Glycemic load (per day)	129.03	134.24	140.12	132.53	0.577
Methylmercury (μg/g)	0.64	0.62	0.72	0.69	0.338
Medication use					
ACEI	36.71	20.17	26.45	26.89	0.421
Diuretics	15.09	13.18	26.46	28.04	0.022
Statins	40.53	30.86	33.79	38.03	0.866
Insulin	4.72	6.18	10.38	0.00	0.019††
Aspirin-antiplatelet drugs	18.30	18.19	17.47	25.84	0.328
CCB	19.16	14.80	13.01	15.01	0.485
Angiotensin II receptor antagonists	20.52	19.43	16.32	11.54	0.155
β-blockers	6.50	14.65	10.33	9.84	0.739

Data given as mean or %. †Adjusted for age, sex and center. ‡Cut-offs for Cr quartiles were 0.3369, 0.6931 and 1.671 μg/g. §Adjusted for sex and center. ¶Adjusted for age and center. ††P for quadratic trend. ACEI, angiotensin-converting enzyme inhibitor; CCB, calcium channel blockers; HDL-C, high-density lipoprotein cholesterol; TCL, toenail chromium level. Other abbreviations as in Table 1.

carried out using Stata 13.0.

Results

Mean subject age was 68.8±6.2 years, and approximately 41% of the population was female. Baseline characteristics are listed in **Table 1**. Compared with controls, patients had a significantly lower adherence to the Mediterranean diet, were more likely to have DM, and had a lower proportion of participants assigned to the Mediterranean diet and nuts PREDIMED arm of the trial. There was no statistically significant difference in TCL between patients and controls.

Table 2 lists the association of TCL with covariates for controls, adjusted for age, sex and center. Compared with those in the lowest quartile of Cr, controls in the highest

Table 3. Multivariable OR for CVD vs. TCL Quartile in PREDIMED Subjects

Variable	TCL quartiles†					
	No. cases/controls	Q1	Q2	Q3	Q4	P for trend
Total sample‡						
Cases/matched controls	147/271	51/68	29/68	40/68	27/67	
Median Cr (μg/g)		0.19	0.45	1.07	2.96	
Matched OR (95% CI)		1 (Ref.)	0.61 (0.35–1.08)	0.83 (0.49–1.43)	0.54 (0.29–1.01)	0.232
Matched OR§ (95% CI)		1 (Ref.)	0.71 (0.38–1.34)	0.90 (0.49–1.68)	0.63 (0.32–1.26)	0.281
Matched OR¶ (95% CI)		1 (Ref.)	0.66 (0.34–1.28)	0.79 (0.41–1.54)	0.54 (0.26–1.14)	0.189
Type 2 DM at baseline††						
Cases/controls	90/133	23/33	22/34	22/33	23/33	
Median Cr (μg/g)		0.14	0.36	0.84	2.61	
Adjusted OR (95% CI)		1 (Ref.)	1.09 (0.48–2.47)	0.89 (0.38–2.06)	1.02 (0.44–2.35)	0.983
Adjusted OR‡‡ (95% CI)		1 (Ref.)	0.90 (0.37–2.19)	0.92 (0.37–2.28)	1.19 (0.50–2.85)	0.569
Adjusted OR§§ (95% CI)		1 (Ref.)	0.83 (0.33–2.07)	0.97 (0.38–2.51)	1.37 (0.54–3.46)	0.364
No type 2 DM at baseline††						
Cases/controls	57/138	22/35	11/34	18/35	6/34	
Median Cr (μg/g)		0.23	0.58	1.23	3.49	
Adjusted OR (95% CI)		1 (Ref.)	0.54 (0.22–1.36)	0.79 (0.34–1.82)	0.28 (0.09–0.82)	0.038
Adjusted OR‡‡ (95% CI)		1 (Ref.)	0.53 (0.21–1.37)	0.80 (0.33–1.94)	0.25 (0.08–0.76)	0.024
Adjusted OR§§ (95% CI)		1 (Ref.)	0.56 (0.21–1.47)	0.72 (0.28–1.87)	0.25 (0.08–0.80)	0.030

†Based on distribution of TCL among controls. ‡Models are from conditional logistic regression analyses with matching factors sex and age. §Adjusted for center (indicator variables), smoking (binary), hypertension, hypercholesterolemia, DM, family history of premature CAD, BMI (continuous) and alcohol intake (3 categories: <5/10 g/day, 5–25/10–50 g/day, >25/50 g/day in women/men). ¶Additionally adjusted for sample mass (continuous), nuts intervention group, baseline adherence to the Mediterranean diet (continuous), physical activity (continuous), total energy intake (continuous), toenail mercury level (continuous), diuretic use (binary) and insulin use (binary). ††Adjusted for sex, age (continuous) and PREDIMED center (indicator variables). ‡‡Additionally adjusted for smoking (binary), hypertension, hypercholesterolemia, family history of premature CAD, BMI (continuous) and alcohol intake (3 categories: <5/10 g/day, 5–25/10–50 g/day, >25/50 g/day in women/men). §§Additionally adjusted for sample mass (continuous), nuts intervention group, baseline adherence to the Mediterranean diet (continuous), physical activity (continuous), total energy intake (continuous), toenail mercury level (continuous), diuretic use (binary) and insulin use (binary). CVD, cardiovascular disease. Other abbreviations as in Tables 1,2.

quartile were younger, more likely to be female, to have no DM, and have diuretic use. We also found that controls in the extreme quartiles (first and fourth) had higher BMI and lower percentage of family history of premature CAD and insulin use than those in the central quartiles of TCL.

Although point estimates for the OR were <1, higher TCL was not significantly associated with decreased CVD risk in this nested case-control study (**Table 3**). After adjusting for recruitment center, smoking, hypertension, hypercholesterolemia, DM, family history of premature CAD, BMI, and alcohol intake, we found no association between TCL and decreased CVD risk (OR, 0.63; 95% CI: 0.32–1.26; P_{trend}=0.281), nor was there any association after further adjusting for sample mass, nuts intervention group, baseline adherence to the Mediterranean diet, physical activity, total energy intake, toenail mercury level, diuretic use and insulin use (OR, 0.54; 95% CI: 0.26–1.14; P_{trend}=0.189). P-values for interaction between TCL and potential effect modifiers were 0.223, 0.342 and 0.078 for age, sex and DM, respectively.

When we stratified by prevalence of DM, in the DM patients we continued to observe no association with CVD risk for the comparison between extreme quartiles of Cr exposure in the unconditional logistic regression model adjusted for age, sex, and recruitment center (OR, 1.02; 95% CI: 0.44–2.35; P_{trend}=0.983), or the fully adjusted unconditional logistic regression model (OR, 1.37; 95% CI: 0.54–3.46; P_{trend}=0.364; **Table 3**). In the non-DM patients, we found a statistically significant graded inverse association between TCL and CVD. Comparing the highest to the lowest quartile, the age-, sex- and recruitment center-adjusted OR was 0.28 (95% CI: 0.09–0.82; P_{trend}=0.038). The inverse association persisted in the fully adjusted unconditional logistic regression model (OR, 0.25; 95% CI: 0.08–0.80; P_{trend}=0.030; **Table 3**).

Tables S1,**S2** show the subgroup analysis for only AMI patients (n=63) and only stroke patients (n=86), respectively. Although non-significant, the pattern observed in the total group (CVD) was still consistent for both separated outcomes, AMI and stroke. In non-DM participants, high TCL was significantly associated with decreased risk of stroke (**Table S2**).

Discussion

Cr exposure, as assessed using an objective long-term integrated biomarker, was inversely associated with CVD in non-DM Spanish adults aged 55–80 years at high CVD risk, but the overall association was not statistically significant and this inverse relationship was not observed in DM patients.

The biological basis for a protective effect of Cr on CVD is relevant. Low Cr is related to elevated triglycerides, total cholesterol, LDL-C, body weight, and reduced high-density lipoprotein cholesterol (HDL-C).[6,11,24,25] Moreover, a recent study in a large sample of US adults noted an association between lower TCL and metabolic syndrome.[11] Despite the number of studies supporting biological plausibility, few epidemiological studies have addressed the relationship between long-term Cr exposure and CVD as

the outcome variable. In the European Multicenter Study on Antioxidants, MI, and Breast Cancer (EURAMIC; a case-control study including 684 men with first MI and 724 men without history of MI, but with similar characteristics), Guallar et al found a clear inverse relationship between TCL and MI.[10] A case-control study conducted in men from the Health Professionals' Follow-up Study (HPFS), also suggested an inverse association between TCL and CVD.[12] The present results extend these findings to men and women without DM and at high risk for CVD, and they increase the limited epidemiological knowledge on the possible protective role of Cr against CVD.

In the DM patients, we found no significant evidence of an association between TCL and CVD. This result is similar to that obtained by Rajpathak et al in a nested case-control study of men from the HPFS.[12] In a secondary analysis, they compared the TCL of 202 men with DM who developed incident CVD with 447 who did not. After adjusting for potential confounders, the OR between extreme quartiles was 1.12 (95% CI: 0.68–1.83; P=0.55). This suggests that, in DM patients, Cr may not be related to CVD. These previous results are in agreement with the lack of association observed in the total sample, given that in the present nested case-control study there was a very high percentage of DM subjects (61.2% in the patient group and 49.1% in controls).

It may be possible that, due to weak associations observed in the total sample and in DM subjects, the statistical power was not sufficient to estimate the effect. According to the observed OR, the statistical power was 57.9% when comparing the highest with the lowest quartile of Cr in the nested case-control study. In the DM patients, the statistical power decreased dramatically: 5% when comparing the highest with the lower quartile of Cr. Despite this, these results may be useful for future meta-analysis when more studies become available.

Concerning non-DM participants, we found a clear inverse association between TCL and CVD. These results cannot be directly compared with previous studies because, to our knowledge, the association between TCL and CVD has not been previously analyzed in non-DM individuals. Given, however, the relatively small percentage of DM subjects in the EURAMIC study (8.4% in the case group and 3.9% in controls),[10] there is a parallel between the present results for non-DM subjects and those from the EURAMIC study: they also found a clear inverse relationship, with an OR between extreme quintiles of 0.59 (95% CI: 0.37–0.95; P_{trend}=0.04) in their fully adjusted model. Further studies are needed to confirm this relationship in non-DM subjects.

A question arises: why is Cr level inversely associated with CVD in non-DM subjects but not in DM subjects? Some facts may help to explain these contrasting findings. First, Cr has been related traditionally to glucose metabolism, hence the scientific literature on Cr effects in non-DM individuals is limited. Second, the benefits of Cr on cardiovascular risk factors have been demonstrated using subjects with Cr supplementation[6,11,23,24] and, hence, with high levels of Cr. Third, DM subjects tend to have lower Cr than non-DM subjects. We found a statistically significant difference in TCL between DM and non-DM subjects: the geometric mean of toenail Cr was 0.59 μg/g in DM subjects and 0.79 μg/g in non-DM subjects (P=0.017).

Based on the aforementioned and the present results, it could be hypothesized that, in DM subjects, Cr participates mainly in the regulation of insulin action rather than in the prevention of CVD. Given that non-DM subjects have proper regulation of glucose metabolism, these subjects could have greater body Cr stores, which could prevent CVD through the mitigation of cardiovascular risk factors. This hypothesis is supported by the recent findings of Bai et al[11] in the Coronary Artery Risk Development in Young Adults (CARDIA) Trace Element Study. They found that TCL was inversely and longitudinally associated with incidence of metabolic syndrome, and this relationship was mainly explained by the association of TCL with blood lipids. Further investigation is needed to confirm this hypothesis.

Although the main source of exposure to Cr in the general population is dietary intake, it is important to analyze the use of Cr-containing supplements in the present participants. Dietary supplement use is widespread in some developed countries. Despite the fact that a meta-analysis of randomized clinical trials found no significant effect of Cr supplementation on glucose concentration in non-DM individuals, and was inconclusive with regard to glycemia control in DM patients,[25] many subjects appear to use Cr supplements to obtain a better control of glycemia. For example, in the National Health and Nutrition Examination Survey (NHANES) for the years 1999–2010, which includes information on 62,160 individuals from the USA, 55.2% of participants reported consuming at least 1 dietary supplement in the previous 30 days, and 26.8% took a supplement that contained Cr.[26] In the present sample, 39 participants (9.33% of the total sample) reported consuming at least 1 dietary supplement in the previous 30 days at baseline, and only 2 of them (0.48% of the total sample) took a supplement that contained Cr. Thus, food was the main source of Cr in the present study.

Valid data on the Cr content of foods are very limited, in part because of a lack of standardized analytical methods.[27] Moreover, the Cr content of foods may increase or decrease due to many factors such as source, processing, and method of preparation. For example, the content of Cr in whole wheat bread is 1.75 μg/g but white bread contains only 0.14 μg/g, and molasses contains 0.27 μg/g but refined sugar 0.02 μg/g.[9] In contrast, acidic foods have been shown to gain Cr content during processing that involves the use of stainless steel containers or utensils. Other dietary components may affect Cr absorption: vitamin C and oxalate enhance Cr absorption but phytate and simple sugars reduce it. Moreover, some drugs such as antacids reduce Cr absorption.[28] Therefore, dietary Cr intake cannot be accurately determined using any existing database. Cr is ubiquitous in the diet, and many foods contribute with trace amounts per serving. Foods with high concentrations of Cr are whole grain products, green beans, broccoli, and brand cereals. Most dairy products, non-processed meats, fish, polished rice and refined flour are poor sources of Cr.[29] In a study on the intake of trace elements via total diet in Spain, Moreiras and Cuadrado found that 68.27% of dietary Cr came from vegetables.[30] Consequently, in addition to the underlying biological mechanisms, high TCL may be associated with diets rich in whole grain products, fruit and vegetables (vitamin C and oxalate), and poor in refined flour and simple sugars, and which enhance the possible protective role of Cr against CVD. In developed countries dietary Cr intake has decreased progressively during the last decades.[27,31,32] It has been argued that this trend toward suboptimal intake of Cr may be associ-

ated with the high incidence of DM and cardiovascular problems occurring in developed countries, particularly in the aging population or those who consume large amounts of processed and sugar-sweetened foods.[10,33]

We acknowledge that there were several limitations in the present study. First, TCL was measured only once at baseline. This single measure may yield random measurement errors, which tend to attenuate risk estimates. As a result, the inverse association of Cr with CVD (observed only in non-DM subjects) is likely to be underestimated. Second, although TCL has been used as a measure of long-term exposure in previous epidemiologic studies,[10–12] a recent study showed that TCL may be compromised by terrestrial contamination.[34] This contamination may explain the positive relationship between mass sample and TCL (**Table 2**). Third, we found higher TCL in women, and this relationship has been observed in another epidemiologic study.[11] The use of nail polish or cosmetics may be an external source of contamination. Nail polish contains Cr and may contaminate nail clippings.[35] Cosmetics contain heavy metals such as Cr, which can be absorbed through the skin.[36] Women in the present study were elderly at baseline (age, 70.6±5.8 years), and the use of nail polish or cosmetics among them was low. In order to ensure minimal contamination (terrestrial or due to cosmetics use), samples were adequately washed before analysis. Fourth, in toenail measurements, we could not differentiate trivalent Cr, which is suggested to be beneficial, from hexavalent Cr that is toxic to human health, and may produce different cardiovascular effects. Last, the present sample was small, and many models were adjusted for many covariates, thus limiting the power, particularly when we adjust rather than match for sex and age in unconditional logistic regression models. It has been empirically demonstrated, however, that the rule of thumb usually suggested (adjusting for 1 confounder for every 10 events) can be relaxed.[37]

Conclusions

In Spanish adults (55–80 years) at high risk of CVD, high TCL might be inversely associated with CVD in non-DM subjects. We did not find any statistically significant association in the DM subjects. These results increase the limited epidemiological knowledge about the possible protective role of Cr against CVD. Diets rich in whole grain products, fruits and vegetables, and poor in refined flour and simple sugars, may be heart-healthy not only for their proven beneficial effects, but also because they increase body Cr stores.

Disclosures

The authors declare no conflict of interest.

Grants

This research was supported by the official funding agency for biomedical research of the Spanish Government, Instituto de Salud Carlos III (ISCIII), through grants provided to research networks specifically developed for the trial (RTIC G03/140 to R.E.; RTIC RD 06/0045 "PREDIMED" to M.A.M-G.), and JR14/00008 to O.C., and through Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBERobn), and by grants from Centro Nacional de Investigaciones Cardiovasculares (CNIC 06/2007), Fondo de Investigación Sanitaria-Fondo Europeo de Desarrollo Regional (Proyecto de Investigación (PI) 04-2239, PI 05/2584, CP06/00100, PI07/0240, PI07/1138, PI07/0954, PI 07/0473, PI10/01407, PI10/02658, PI11/01647, P11/02505 and PI13/00462), Ministerio de Ciencia e Innovación (Recursos y teconologia agroalimentarias (AGL)-2009-13906-C02 and AGL2010-22319-C03 and AGL2013-49083C3-1-R), Fundación Mapfre 2010, Consejería de Salud de la Junta de Andalucía (PI0105/2007), Public Health Division of the Department of Health of the Autonomous Government of Catalonia, Generalitat Valenciana (Generalitat Valenciana Ayuda Complementatia GVACOMP) 06109, GVACOMP2010-181, GVACOMP2011-151), Conselleria de Sanitat y AP; Atención Primaria (CS) 2010-AP-111, and CS2011-AP-042), Regional Government of Navarra (P27/2011), and Centre Català de la Nutrició de l'Institut d'Estudis Catalans. Hojiblanca and Patrimonio Communal Olivarero donated extra-virgin olive oil; the California Walnut Commission donated walnuts; Borges donated almonds; La Morella Nuts donated hazelnuts. The funding sources had no role in the design and conduct of the study; collection, management, analysis, or interpretation of the data; or preparation, review, or approval of the manuscript.

References

1. Schwarz K, Mertz W. Chromium (III) and the glucose tolerance factor. *Arch Biochem Biophys* 1959; **85:** 292–295.
2. Jeejeebhoy KN, Chu RC, Marliss EB, Greenberg GR, Bruce-Robertson A. Chromium deficiency, glucose intolerance, and neuropathy reversed by chromium supplementation, in a patient receiving long-term total parenteral nutrition. *Am J Clin Nutr* 1977; **30:** 531–538.
3. Mertz W. Chromium in human nutrition: A review. *J Nutr* 1993; **123:** 626–633.
4. Vincent JB. The nutritional biochemistry of chromium (III). Amsterdam: Elsevier, 2007.
5. Anderson RA. Chromium as an essential nutrient for humans. *Regul Toxicol Pharmacol* 1997; **26:** S35–S41.
6. Cefalu WT, Hu FB. Role of chromium in human health and in diabetes. *Diabetes Care* 2004; **27:** 2741–2751.
7. EFSA Panel on Dietetic Products Nutrition and Allergies (NDA). Scientific opinion on dietary reference values for chromium. *EFSA J* 2014; **12:** 3845.
8. Di Bona KR, Love S, Rhodes NR, McAdory D, Sinha SH, Kern N, et al. Chromium is not an essential trace element for mammals: Effects of a low-chromium diet. *J Biol Inorg Chem* 2011; **16:** 381–390.
9. Schroeder HA. The role of chromium in mammalian nutrition. *Am J Clin Nutr* 1968; **21:** 230–244.
10. Guallar E, Jiménez FJ, van't Veer P, Bode P, Riemersma RA, Gómez-Aracena J, et al. Low toenail chromium concentration and increased risk of nonfatal myocardial infarction. *Am J Epidemiol* 2005; **162:** 157–164.
11. Bai J, Xun P, Morris S, Jacobs DR, Liu K, He K. Chromium exposure and incidence of metabolic syndrome among American young adults over a 23-year follow-up: The CARDIA Trace Element Study. *Sci Rep* 2015; **5:** 15606.
12. Rajpathak S, Rimm EB, Li T, Morris JS, Stampfer MJ, Willett WC, et al. Lower toenail chromium in men with diabetes and cardiovascular disease compared with healthy men. *Diabetes Care* 2004; **27:** 2211–2216.
13. Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, Ruiz-Gutiérrez V, Covas MI, et al. Effects of a Mediterranean-style diet on cardiovascular risk factors: A randomized trial. *Ann Intern Med* 2006; **145:** 1–11.
14. Salas-Salvadó J, Garcia-Arellano A, Estruch R, Marquez-Sandoval F, Corella D, Fiol M, et al. Components of the Mediterranean-type food pattern and serum inflammatory markers among patients at high risk for cardiovascular disease. *Eur J Clin Nutr* 2008; **62:** 651–659.
15. The Thematic Network. PREDIMED trial. http://www.predimed.es/ (accessed May 20, 2016).
16. He K. Trace elements in nails as biomarkers in clinical research. *Eur J Clin Invest* 2011; **41:** 98–102.
17. Blaauw M. The holistic analysis of gamma-ray spectra in instrumental neutron activation analysis. *Nucl Instruments Methods Phys Res Sect A Accel Spectrometers Detect Assoc Equip* 1994; **353:** 269–271.
18. Bode P, Blaauw M. Performance and robustness of a multi-user, multi-spectrometer system for INAA. *J Radioanal Nucl Chem* 2012; **291:** 299–305.
19. Schröder H, Fitó M, Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, et al. A short screener is valid for assessing Mediterranean diet adherence among older Spanish men and women. *J Nutr* 2011; **141:** 1140–1145.
20. Elosua R, Marrugat J, Molina L, Pons S, Pujol E. Validation of

the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men: The MARATHOM Investigators. *Am J Epidemiol* 1994; **139:** 1197–1209.
21. Martínez-González MA, García-Arellano A, Toledo E, Salas-Salvadó J, Buil-Cosiales P, Corella D, et al. A 14-item Mediterranean diet assessment tool and obesity indexes among high-risk subjects: The PREDIMED trial. *PLoS One* 2012; **7:** e43134.
22. Fernández-Ballart JD, Piñol JL, Zazpe I, Corella D, Carrasco P, Toledo E, et al. Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *Br J Nutr* 2010; **103:** 1808–1816.
23. Sharma S, Agrawal RP, Choudhary M, Jain S, Goyal S, Agarwal V. Beneficial effect of chromium supplementation on glucose, HbA1C and lipid variables in individuals with newly onset type-2 diabetes. *J Trace Elem Med Biol* 2011; **25:** 149–153.
24. Pittler MH, Stevinson C, Ernst E. Chromium picolinate for reducing body weight: Meta-analysis of randomized trials. *Int J Obes Relat Metab Disord* 2003; **27:** 522–529.
25. Balk EM, Tatsioni A, Lichtenstein AH, Lau J, Pittas AG. Effect of chromium supplementation on glucose metabolism and lipids: A systematic review of randomized controlled trials. *Diabetes Care* 2007; **30:** 2154–2163.
26. McIver DJ, Grizales AM, Brownstein JS, Goldfine AB. Risk of type 2 diabetes is lower in US adults taking chromium-containing supplements. *J Nutr* 2015; **145:** 2675–2682.
27. Stoecker BJ. Basis for dietary recommendations for chromium. *In*: Vincent JB, editor. The nutritional biochemistry of chromium (III). Amsterdam: Elsevier, 2007; 43–55.
28. Otten JJ, Hellwig JP, Meyers L, editors. Dietary reference intakes: The essential guide to nutrient requirements. Washington, DC: National Academies Press, 2006.
29. Anderson RA, Bryden NA, Polansky MM. Dietary chromium intake: Freely chosen diets, institutional diet, and individual foods. *Biol Trace Elem Res* 1992; **32:** 117–121.
30. Moreiras O, Cuadrado C. Theoretical study of the intake of trace elements (nutrients and contaminants) via total diet in some geographical areas of Spain. *Biol Trace Elem Res* 1992; **32:** 93–103.
31. Anderson RA, Kozlovsky AS. Chromium intake, absorption and excretion of subjects consuming self-selected diets. *Am J Clin Nutr* 1985; **41:** 1177–1183.
32. Kumpulainen JT. Chromium content of foods and diets. *Biol Trace Elem Res* 1992; **32:** 9–18.
33. Anderson RA. Nutritional role of chromium. *Sci Total Environ* 1981; **17:** 13–29.
34. Hashemian M, Poustchi H, Pourshams A, Khoshnia M, Brockman JD, Hekmatdoost A, et al. The Nail as a Biomonitor of Trace Element Status in Golestan Cohort Study. *Middle East J Dig Dis* 2016; **8:** 19–23.
35. Favaro PC, Bode P, De Nadai Fernandes EA. Trace elements in nail polish as a source of contamination of nail clippings when used in epidemiological studies. *J Radioanal Nucl Chem* 2005; **264:** 61–65.
36. Borowska S, Brzóska MM. Metals in cosmetics: Implications for human health. *J Appl Toxicol* 2015; **35:** 551–572.
37. Vittinghoff E, McCulloch CE. Relaxing the rule of ten events per variable in logistic and Cox regression. *Am J Epidemiol* 2007; **165:** 710–718.

Supplementary Files

Supplementary File 1

Table S1. Multivariable OR for MI vs. TCL quartile in PREDIMED subjects

Table S2. Multivariable OR for stroke vs. TCL quartile in PREDIMED subjects

Please find supplementary file(s);
http://dx.doi.org/10.1253/circj.CJ-17-0032