Chromium Exposure and Risk of Cardiovascular Disease in High Cardiovascular Risk Subjects - Nested Case-Control Study in the Prevention With Mediterranean Diet (PREDIMED) Study.

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ABSTRACT

Background

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

Methods and Results

We evaluated associations between TCL and CVD in a case-control study nested within the "PREvención con DIeta MEDiterránea" (PREDIMED) trial. We randomly selected 147 of the 288 cases 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 odds ratio (OR) for the highest versus lowest quartile of toenail chromium was 0.54 (95% Confidence Interval [CI], 0.26, 1.14; p_{trend}=0.189) for the nested case-control study. When we stratified by diabetes, the ORs were 1.37 (95% CI, 0.54, 3.46; p_{trend}=0.364) within the diabetic group, and 0.25 (95% CI, 0.08, 0.80; p_{trend}=0.030) within the non-diabetic one (p for interaction=0.078).

Conclusions

Our findings, though not statistically significant, are consistent with previously reported inverse associations between TCL and CVD. These results, especially among non-diabetics, increase the limited epidemiological knowledge about the possible protective role of chromium against CVD.

Trial registration: www.controlled-trials.com; ISRCTN35739639.

Keywords: chromium; cardiovascular diseases; toenail biomarker; diabetes; PREDIMED

INTRODUCTION

Chromium is one of the most common elements in the earth crush and seawater, and is found in the environment primarily in two valence states, trivalent (+3) chromium and hexavalent (+6) chromium. Trivalent chromium is an essential trace element present in most foods and with very low toxicity. Hexavalent chromium is much less abundant, highly toxic for humans, and largely synthesized by the oxidation of trivalent chromium. The nutritional importance of trivalent chromium started in the 1950s when the need of chromium to maintain normal glucose tolerance in rats was reported[1]. In 1977 the essentiality of chromium 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 the intravenous administration of trivalent chromium[2]. Through the next decades, the requirements of chromium for normal carbohydrate, lipid and protein metabolism were established[3–6]. However, the essentiality of chromium has been questioned in recent years due to the failure to identify the underlying biological mechanisms to explain its action[7,8].

Despite the fact that more than 40 years ago Schroeder[9] hypothesized that chromium deficiency represented a significant risk factor for cardiovascular disease (CVD), epidemiological data on chromium intake and the risk of CVD are still limited. One limitation is the use of serum or urinary chromium measurements, which may not adequately reflect long-term exposure. Several studies have used toenail chromium levels (TCL) as a measure of long-term chromium exposure[10–13], and three of them assessed cardiovascular endpoints[10,12,13]. These three studies were conducted in men only, so their findings cannot be generalized to women. However, all the above studies suggested that chromium might play a protective role against CVD.

The purpose of this study was to assess the hypothesis that long-term chromium 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 PREDIMED (PREvención con DIeta MEDiterránea) study.

METHODS

Study Design

The design and methods of the PREDIMED trial have been described previously[14,15]. The PREDIMED trial (www.predimed.es) was a randomized, controlled, CVD prevention trial based in 11 centers throughout Spain[16]. Institutional Review Boards at all participating centers approved the study protocol. The study began in October 2003 and it was stopped because of early benefit by December 1, 2010.

Eligible participants included men (55 to 80 years) and women (60 to 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 or at least three of the following major risk factors: current smoking, hypertension, elevated low-density lipoprotein cholesterol levels, overweight or obesity, or a family history of premature coronary heart disease (CHD). 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.

Cases and Controls

Among the 7,447 participants of PREDIMED, 7,232 (97.1%) of them provided toenail clippings at baseline, within six months of randomization. Among them, we randomly selected 147 of the 288 cases of incident CVD. The median follow-up period from time of toenail sampling to time of incident CVD was 4.8 years (interquartile range 3.0 to 5.8 years). Outcomes were identified through repeated contacts with participants, contacts

with family physicians, annual medical record review, and consultation of the National Death Index.

Cases were randomly matched on age (within two years) and sex to 271 controls who were free from CVD before December 2010 and had provided toenail samples at baseline. Most cases were matched to two controls, but 23 cases were matched to only one 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. Toenail reflects body intake or exposure for a time frame from a few months to a year[17]. 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 by 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 analysed along with the rest to safeguard against chromium contamination in the analysis process. A precise description of the analytical methodology has been published elsewhere[18,19].

Chromium 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 measurement by INAA is reproducible to 0.1% from date to date. In each series of samples, a reference material was incorporated (INCT-PVTL-6). This material only has a recommended value for Cr. However, the laboratory for INAA monitor their neutron spectrum with a flux monitor containing Cr that is directly traceable to the NIST standard solution that it is used to prepare the flux monitors with: SRM 3112a - Chromium (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 s.d.) across 9 reference materials covering certified values from 0.05 to 200 mg/kg. Along the year, 400 of these analyses are repeated.

Covariate Assessment

All the covariates were measured at baseline and yearly over follow-up. We reviewed medical records and used standardized validated protocols[20] to collect information on sociodemographic, lifestyle, health, family history, medication use and medical diagnoses. We grouped medications used habitually by participants at baseline into eight categories: Angiotensin converting enzyme inhibitors, Diuretics, Statins, Insulin, Aspirin-antiplatelet drugs, Calcium channel blockers, Angiotensin II receptors antagonists and Beta-blockers. A validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire[21] was used to evaluate physical activity. Trained nurses measured 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[22] 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 diabetes 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

We used the means (standard deviations) and percentages to describe characteristics of the study population for continuous and categorical variables, respectively. Group

comparisons were made using t-test or chi-squared test as appropriate. TCL (µg/g) were categorized into quartiles based on the distribution among controls.

Adjusted levels of covariates across quartiles of chromium in controls were estimated by ANOVA. Whether these adjusted levels were associated with quartiles of chromium 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, matching on age and sex. We investigated possible interactions between TCL and potential effect modifiers (age, sex and diabetes) by adding a corresponding multiplicative interaction term in the models, followed by the likelihood ratio test.

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

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

We estimated the odds ratios (ORs) and 95% confidence intervals (CIs) using the lowest quartile as the reference category. Tests for trend were performed by assigning each subject the median value of the quartile of chromium and treating it as a continuous variable. We also used this variable to create the multiplicative interaction terms above 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, diabetes, family history of premature heart disease, body mass index, alcohol intake,

sample mass, intervention group, baseline adherence to Mediterranean diet, physical activity, total energy intake, toenail methilmercury level, diuretic use and insulin use.

All p-values reported are two-tailed, and values below 0.05 were considered statistically significant. We performed all statistical analyses using Stata 13.0 software.

RESULTS

The mean (SD) age of all participants was 68.8 (6.2) years, and approximately 41% of the population was female. Baseline characteristics for cases and controls are shown in Table 1. Compared with controls, cases had a significantly lower adherence to the Mediterranean diet, were more likely to be diabetic, 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 cases and controls.

Table 2 shows the association of TCL with covariates among controls, adjusted for age, sex and center. Compared with those in the lowest quartile of chromium, controls in the highest quartile were younger, more likely to be female, non-diabetic and diuretic users. We also found that controls in the extreme quartiles (first and fourth) had higher body mass index (BMI) and lower percentage of family history of premature CHD and insulin use than those in the central quartiles of TCL.

Though point estimates for the OR were lower than 1, higher TCL was not significantly associated with decreased CVD risk in our nested case-control study (Table 3). After adjusting for recruitment center, smoking, hypertension, hypercholesterolemia, diabetes, family history of premature CHD, BMI, and alcohol intake, we found no association (OR, 0.63; 95% CI, 0.32, 1.26; p_{trend}=0.281), as did results 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 diabetes respectively.

When we stratified by prevalent diabetes, among diabetic patients we continued to observe no association with CVD risk for the comparison between extreme quartiles of chromium exposure in 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 fully-adjusted unconditional logistic regression model (OR, 1.37; 95% CI, 0.54, 3.46; p_{trend}=0.364) (Table 3). In non-diabetic 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).

Supplemental tables 1 and 2 show the subgroup analyses of only cases with AMI (n=63) and only cases with stroke (n=86), respectively. Though non-significant, the pattern observed in the total group (CVD) was still consistent for both separated outcomes, AMI and stroke. In non-diabetic participants, high TCL were significantly associated with decreased risk of stroke (Supplemental Table 2).

DISCUSSION

Our results indicate that chromium exposure, as assessed by an objective long-term integrated biomarker, was inversely associated with CVD among non-diabetic Spanish adults aged 55-80 years at high CVD risk. However, the overall association was not statistically significant and this inverse relationship was not observed among diabetic patients.

The biological bases for a protective effect of chromium on CVD are relevant. Low chromium levels are related with elevated levels of triglycerides, total cholesterol, low density lipoprotein cholesterol (LDL), body weight, and reduced levels of high density lipoprotein cholesterol (HDL)[6,11,24,25]. Moreover, a recent study in a large sample of American adults has related lower TCL with metabolic syndrome[11]. Despite the number of studies supporting biological plausibility, few epidemiological studies have addressed the relationship between long-term chromium exposure and CVD as the outcome variable. Guallar *et al.*[10] in the European Multicenter Study on Antioxidants, MI, and Breast Cancer (EURAMIC) (a case-control study including 684 men with a first MI and 724 men without history of MI, but with similar characteristics), found a clear inverse relationship between TCL and MI. Two case-control studies conducted in men from the Health Professionals' Follow-up Study (HPFS), also suggested an inverse association between TCL and CVD[12,13]. Our results extend the above findings to men and women without diabetes and at high risk for CVD, and they increase the limited epidemiological knowledge about the possible protective role of chromium against CVD.

Among diabetics, we found no significant evidence of association between TCL and CVD. Our results are similar to those obtained by Rajpathak *et al.*[12] in a nested case-control study of men from the HPFS. In a secondary analysis, they compared the TCL of 202 diabetic men 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). These findings suggest that, among diabetics, chromium levels may not be related to CVD. These previous results are in consistency with the lack of association observed in the total sample since, in our nested case-control study, we had a very high percentage of diabetic patients (61.2% in cases and 49.1% in controls).

It may be possible that, due to weak associations observed in the total sample and among diabetics, there is not enough statistical power to estimate the effect. According

to the observed ORs, the statistical power was 57.9% when comparing the highest with the lowest quartile of chromium in the nested case-control study. Among diabetic patients, the statistical power decreased dramatically: 5% when comparing the highest with the lower quartile of chromium. Notwithstanding the foregoing, these results may be useful for future meta-analysis when more studies become available.

Concerning non-diabetic participants, we found a clear inverse association between TCL and CVD. Our results cannot be directly compared to previous studies because, to our knowledge, the association between TCL and CVD has not been previously analyzed in non-diabetic individuals. However, given the relatively small percentage of diabetics in the EURAMIC study sample (8.4% in cases and 3.9% in controls) [10], we could establish a parallelism between our results among non-diabetics 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-diabetics.

A question arises from our results: why chromium levels showed an inverse association with CVD in non-diabetics but not in diabetics? Some facts may help us to explain these contrasting findings. First, chromium has been related traditionally to glucose metabolism and so, the scientific literature about chromium effects in non-diabetic individuals is limited. Second, the benefits of chromium on cardiovascular risk factors have been shown in subjects with chromium supplementation [6,11,24,25] and therefore, with high levels of chromium. Third, diabetics tend to have lower levels of chromium than non-diabetics ones. We found statistically significant differences in TCL between diabetics and non-diabetics: geometric means of toenail chromium were 0.59 and 0.79 μg/g in diabetics and non-diabetics respectively (p= 0.017).

Based on the above and our results, it could be hypothesized that, among diabetics, chromium participates mainly in the regulation of insulin action rather than in the

prevention of CVD. Since non-diabetics have proper regulation of glucose metabolism, these subjects could have greater body chromium stores, which could prevent CVD through the mitigation of cardiovascular risk factors. Our hypothesis is supported by the recent findings reported by Bai *et al.*[11] in the Coronary Artery Risk Development in Young Adults (CARDIA) Trace Element Study. They found that TCL were 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 chromium in the general population is dietary intake, it is important to analyze the use of chromium-containing supplements in our 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 chromium supplementation on glucose concentrations in non-diabetic individuals, and was inconclusive with regard to glycaemia control among diabetic patients[26], many subjects appear to use chromium supplements to obtain a better control of glycaemia. 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 United States, 55.2% of participants reported consuming at least one dietary supplement in the previous 30 days, and 26.8% took a supplement that contained chromium[27]. In our sample, 39 participants (9.33% of the total sample) reported consuming at least one 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 chromium. Thus, food was the main source of chromium in our study.

Valid data on the chromium content of foods are very limited, in part because of a lack of standardized analytical methods[28]. Moreover, the chromium content of foods may increase or decrease due to many factors such as source, processing, and method of

preparation. For example, the content of chromium 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]. On the other hand, acidic foods have been shown to gain chromium content during processing that involves the use of stainless steel containers or utensils. Other dietary components may affect chromium absorption: vitamin C and oxalate enhance chromium absorption but phytate and simple sugars reduce it. Moreover, some drugs such as antacids reduce chromium absorption[29]. Therefore, dietary chromium intake cannot be accurately determined using any existing database. Chromium is ubiquitous in the diet, and many foods contribute with trace amounts per serving. Foods with high concentrations of chromium 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 chromium[30]. In a study on the intake of trace elements via total diet in Spain, Moreiras *et al.*[31] found that 68.27% of dietary chromium came from vegetables. 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, which enhance the possible protective role of chromium against CVD. In developed countries dietary chromium intakes have decreased progressively during the last decades[28,32,33]. It has been argued that this trend toward suboptimal intake of chromium may be associated with the high incidence of diabetes and cardiovascular problems occurring in developed countries, particularly in aging populations or populations who consume large amounts of processed and sugar-sweetened foods[10,34].

We acknowledge that there were several limitations in our 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

chromium with CVD (observed only among non-diabetics) 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[35]. This contamination may explain the positive relationship between mass sample and TCL (see 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 polishes contains chromium and they may contaminate nail clippings[36]. Cosmetics have heavy metals like chromium, which can be absorbed through the skin[37]. Women in our sample were elderly at baseline (mean age = 70.6 years; standard deviation = 5.8 years), and the use of nail polishes or cosmetics among them was scarce. In order to ensure minimal contamination (terrestrial or due to cosmetics use), samples were adequately washed before the analysis. Fourth, in toenail measurements, we could not differentiate trivalent chromium, which is suggested to be beneficial, from hexavalent chromium that is toxic for human health, and may produce different cardiovascular effects. Lastly, our sample size is small and many models are adjusted for many covariates, thus limiting our power, particularly when we adjust rather than match for sex and age in unconditional logistic regression models. However, it has been empirically demonstrated that the rule of thumb usually suggested (adjusting for one confounder for every 10 events) can be relaxed[38].

In conclusion, we have investigated the association between TCL and CVD in Spanish adults (55-80 years) at high risk of CVD. Our findings suggest that high TCL might be inversely associated with CVD among non-diabetics. We did not find any statistically significant association among diabetics. These results increase the limited epidemiological knowledge about the possible protective role of chromium against CVD. Diets rich in whole grains 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 chromium stores.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Schwarz K, Mertz W. Chromium(III) and the glucose tolerance factor. Arch Biochem Biophys [Internet] 1959 [cited 2016 Oct 25];**85**:292–295. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14444068
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 [Internet] 1977 [cited 2016 Oct 25];**30**(4):531–538. Available from: http://www.ncbi.nlm.nih.gov/pubmed/192066
3. Mertz W. Chromium in human nutrition: a review. J Nutr [Internet] 1993 [cited 2016 Oct 26];**123**(4):626–633. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8463863
4. Vincent JB. The nutritional biochemistry of chromium (III). Elsevier; 2007.
5. Anderson RA. Chromium as an essential nutrient for humans. Regul Toxicol Pharmacol [Internet] 1997 [cited 2016 Oct 26];**26**(1 Pt 2):S35-S41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9380836
6. Cefalu WT, Hu FB. Role of chromium in human health and in diabetes. Diabetes Care [Internet] 2004 [cited 2016 Jun 7];**27**(11):2741–2751. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15505017
7. EFSA Panel on Dietetic Products Nutrition and Allergies (NDA). Scientific Opinion on Dietary Reference Values for chromium. EFSA J [Internet] 2014 [cited 2016 Oct 18];**12**(10):3845. Available from: http://doi.wiley.com/10.2903/j.efsa.2014.3845
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 [Internet] 2011 [cited 2016 Oct 26];**16**(3):381–390. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21086001
9. Schroeder HA. The role of chromium in mammalian nutrition. Am J Clin Nutr [Internet] 1968 [cited 2016 Oct 26];**21**(3):230–244. Available from: http://www.ncbi.nlm.nih.gov/pubmed/5642900
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 [Internet] 2005 [cited 2016 Mar 22];**162**(2):157–164. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15972934
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 [Internet] 2015 [cited 2016

May 23];**5**:15606. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4614983&tool=pmcentrez&rendertype=abstract
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 [Internet] 2004 [cited 2016 Mar 22];**27**(9):2211–2216. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15333486
13. Rimm E, Giovannucci E, Ascherio A, Stampfer M, Willett W, Hu F. Toenail chromium levels and risk of coronary heart disease among normal and overweight men. In: AHA- Epidemiology Meeting Abstract. 2002.
14. 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 [Internet] 2006 [cited 2016 Apr 9];**145**(1):1–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16818923
15. 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 [Internet] 2008 [cited 2016 Apr 16];**62**(5):651–659. Available from: http://dx.doi.org/10.1038/sj.ejcn.1602762
16. Investigators TP. The Thematic Network - Predimed.es [Internet]. [cited 2016 May 20];Available from: http://www.predimed.es/the-thematic-network.html
17. He K. Trace elements in nails as biomarkers in clinical research. Eur J Clin Invest [Internet] 2011 [cited 2017 Feb 14];**41**(1):98–102. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20813017
18. 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 [Internet] 1994 [cited 2017 Feb 14];**353**(1–3):269–271. Available from: http://linkinghub.elsevier.com/retrieve/pii/0168900294916543
19. Bode P, Blaauw M. Performance and robustness of a multi-user, multi-spectrometer system for INAA. J Radioanal Nucl Chem [Internet] 2012 [cited 2017 Feb 14];**291**(2):299–305. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26224912
20. 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 [Internet] 2011 [cited 2016 Mar 1];**141**(6):1140–1145. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21508208
21. 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 [Internet] 1994 [cited 2016 Mar 24];**139**(12):1197–1209. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8209878
22. 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 [Internet] 2012 [cited 2016 Apr 2];**7**(8):e43134. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3419206&tool=pmcentrez&rendertype=abstract
23. 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 [Internet] 2010 [cited 2015 Dec

6];**103**(12):1808–1816. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20102675
24. 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 [Internet] 2011 [cited 2016 Jun 28];**25**(3):149–153. Available from: http://www.sciencedirect.com/science/article/pii/S0946672X11000472
25. Pittler MH, Stevinson C, Ernst E. Chromium picolinate for reducing body weight: meta-analysis of randomized trials. Int J Obes Relat Metab Disord [Internet] 2003 [cited 2016 Jun 9];**27**(4):522–529. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12664086
26. 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 [Internet] 2007;**30**(8):2154–2163. Available from: http://care.diabetesjournals.org/cgi/doi/10.2337/dc06-0996
27. McIver DJ, Grizales AM, Brownstein JS, Goldfine AB. Risk of Type 2 Diabetes Is Lower in US Adults Taking Chromium-Containing Supplements. J Nutr [Internet] 2015 [cited 2016 Jun 7];**145**(12):2675–2682. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26446484
28. Stoecker BJ. Basis for dietary recommendations for chromium. In: Vincent JB, editor. The Nutritional Biochemistry of Chromium (III). Elsevier; 2007. p. 43–55.
29. Otten JJ, Hellwig JP, Meyers L, editors. Dietary Reference Intakes : The Essential Guide to Nutrient Requirements. Washington, DC: National Academies Press; 2006.
30. Anderson RA, Bryden NA, Polansky MM. Dietary chromium intake: freely chosen diets, institutional diet, and individual foods. Biol Trace Elem Res [Internet] 1992 [cited 2016 Oct 16];**32**(1–3):117–121. Available from: http://link.springer.com/10.1007/BF02784595
31. 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 [Internet] [cited 2016 Oct 23];**32**:93–103. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1375092
32. Anderson RA, Kozlovsky AS. Chromium intake, absorption and excretion of subjects consuming self-selected diets. Am J Clin Nutr [Internet] 1985 [cited 2016 Oct 23];**41**(6):1177–1183. Available from: http://www.ncbi.nlm.nih.gov/pubmed/4003325
33. Kumpulainen JT. Chromium content of foods and diets. Biol Trace Elem Res [Internet] [cited 2016 Oct 24];**32**:9–18. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1375091
34. Anderson RA. Nutritional role of chromium. Sci Total Environ [Internet] 1981 [cited 2016 Oct 23];**17**(1):13–29. Available from: http://linkinghub.elsevier.com/retrieve/pii/0048969781901042
35. 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 [Internet] 2016 [cited 2016 Apr 27];**8**(1):19–23. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4773078&tool=pmcentrez&rendertype=abstract
36. 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 [Internet] 2005 [cited 2016 May 23];**264**(1):61–65. Available

from: http://link.springer.com/10.1007/s10967-005-0675-z
37. Borowska S, Brzóska MM. Metals in cosmetics: implications for human health. J Appl Toxicol [Internet] 2015 [cited 2016 May 23];**35**(6):551–572. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25809475
38. Vittinghoff E, McCulloch CE. Relaxing the rule of ten events per variable in logistic and Cox regression. Am J Epidemiol [Internet] 2007 [cited 2016 Oct 24];**165**(6):710–718. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17182981

Table 1. Baseline Characteristics of Case Participants with Incident Cardiovascular Disease and Matched Controls from the PREDIMED study.

Characteristic	Case participants	Control participants	p value
n	147	271	
Age (median, IQR)	70 (64-75)	69 (64-74)	Matching Factor
Gender (% women)	40.3	40.5	Matching Factor
Toenail chromium[1] (μg/g)	0.61	0.72	0.215
Toenail methilmercury[1] (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 diabetes (%)	61.2	49.1	0.017
Family history of CHD (%)	19.7	15.5	0.271
Body mass index (kg/m^2)	29.7 (3.6)	29.7 (3.5)	0.937
Physical activity (METs-min/d)	250.2 (211.1)	279.0 (272.5)	0.267
Alcohol (g/d)	10.1 (16.8)	12.7 (17.9)	0.150
Dietary intake			
Total energy intake (kcal/d)	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/d)	363.0 (122.2)	366.5 (130.9)	0.784
Fiber (g/d)	24.9 (10.3)	25.1 (7.9)	0.784
Mediterranean diet adherence (0 to 14)	8.2 (2.0)	8.8 (1.8)	0.001

Means (standard deviations) unless otherwise stated. IQR: interquartile range. EVOO: extra-virgin olive oil. CHD: coronary heart disease METs: metabolic equivalents. %E: percentage of total energy intake. OR: Odds ratio. CI: Confidence Interval. [1]Geometric means.

Table 2. Adjusted[1] baseline characteristics of 271 healthy controls by quartiles of toenail chromium levels.

Variables	Quartiles[2] of toenail chromium				
	Q1	Q2	Q3	Q4	p for linear trend
No. of participants	68	68	68	67	
Median chromium level (μg/g)	0.18	0.45	1.09	2.90	NA
Age[3] (years)	70.08	68.19	68.81	67.39	0.024
Female sex[4] (%)	25.69	39.32	38.00	58.14	<0.001
Toenail mass (mg)	54.74	62.08	72.70	70.15	0.050
Body mass index (kg/m^2)	30.12	29.06	29.25	30.34	0.011[5]
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 diabetes (%)	61.79	48.98	40.78	44.70	0.031
Family history of CHD (%)	11.19	20.52	19.20	11.02	0.049[5]
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 cholesterol (mg/dL)	53.81	55.76	50.65	55.50	0.997
Mediterranean diet adherence (0 to 14)	8.92	8.92	8.95	8.56	0.296
Dietary intake					
Total energy intake (kcal/day)	2330.24	2366.56	2396.35	2359.73	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	129.03	134.24	140.12	132.53	0.577
Methilmercury level (μg/g)	0.64	0.62	0.72	0.69	0.338
Medication use (%)					
ACE inhibitors	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[5]
Aspirin-antiplatelet drugs	18.30	18.19	17.47	25.84	0.328
Calcium channel blockers	19.16	14.80	13.01	15.01	0.485
Angiotensin II receptor antagonists	20.52	19.43	16.32	11.54	0.155
Beta-blockers	6.50	14.65	10.33	9.84	0.739

Data are means, unless otherwise specified. EVOO: extra-virgin olive oil. CHD: coronary heart disease. METs: metabolic equivalents. %E: percentage of total energy intake. ACE: Angiotensin converting enzyme inhibitors. [1]Adjusted for age, sex and center. [2]Cut-off values for quartiles of chromium were 0.3369, 0.6931 and 1.671 μg/g. [3]Adjusted for sex and center. [4]Adjusted for age and center. [5]p for quadratic trend

Table 3. Center-Adjusted and multivariable odds ratios for cardiovascular disease (CVD) by quartile of baseline toenail chromium level among CVD cases and matched controls, the PREDIMED trial.

Variable		Quartiles[1] of toenail chromium				
	number of cases/controls	Q1	Q2	Q3	Q4	p for trend
Total sample[2]						
Cases / matched controls	147/271	51/68	29/68	40/68	27/67	
Median of 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[3] (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[4] (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 diabetes at baseline[5]						
Cases / controls	90/133	23/33	22/34	22/33	23/33	
Median of 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[6] (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[7] (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 diabetes at baseline[5]						
Cases / controls	57/138	22/35	11/34	18/35	6/34	
Median of 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[6] (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[7] (95% CI)		1 (ref.)	0.56(0.21-1.47)	0.72(0.28-1.87)	0.25(0.08-0.80)	0.030

[1]Quartiles based on distribution of toenail chromium among controls. [2]Models are from conditional logistic regression analyses with matching factors sex and age. [3]Adjusted for center (indicator variables), smoking (binary), hypertension, hypercholesterolemia, diabetes, family history of premature coronary heart disease, body mass index (continuous) and alcohol intake (three categories: <5/10 g/day, 5-25/10-50 g/day, >25/50 g/day in women/men). [4]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). [5]Adjusted for sex, age (continuous) and PREDIMED center (indicator variables). [6]Additionally adjusted for smoking (binary), hypertension, hypercholesterolemia, family history of premature coronary heart disease, body mass index (continuous) and alcohol intake (three categories: <5/10 g/day, 5-25/10-50 g/day, >25/50 g/day in women/men). [7]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).

Supplemental Table 1. Center-Adjusted and multivariable odds ratios for myocardial infarction (MI) by quartile of baseline toenail chromium level among MI cases and controls, the PREDIMED trial.

Variable		Quartiles[1] of toenail chromium				
	number of cases/controls	Q1	Q2	Q3	Q4	p for trend
Total sample[2]						
Cases / controls	63/271	20/68	14/68	16/68	13/67	
Median of Cr (μg/g)		0.19	0.45	1.07	2.96	
Adjusted OR (95% CI)		1 (ref.)	0.87(0.39-1.95)	0.73(0.32-1.64)	0.74(0.31-1.76)	0.534
Adjusted OR[3] (95% CI)		1 (ref.)	0.87(0.38-2.03)	0.79(0.33-1.86)	0.76(0.31-1.85)	0.588
Adjusted OR[4] (95% CI)		1 (ref.)	0.83(0.35-1.95)	0.74(0.30-1.83)	0.68(0.27-1.74)	0.481
Type 2 diabetes at baseline[2]						
Cases / controls	39/133	9/33	11/34	10/33	9/33	
Median of Cr (μg/g)		0.14	0.36	0.84	2.61	
Adjusted OR (95% CI)		1 (ref.)	1.61(0.48-2.47)	0.83(0.38-2.06)	0.93(0.44-2.35)	0.667
Adjusted OR[5] (95% CI)		1 (ref.)	1.07(0.29-4.01)	0.87(0.23-3.24)	1.08(0.29-4.04)	0.906
Adjusted OR[6] (95% CI)		1 (ref.)	1.18(0.29-4.79)	1.14(0.28-4.72)	1.53(0.36-6.57)	0.578
No type 2 diabetes at baseline[2]						
Cases / controls	24/138	8/35	7/34	6/35	3/34	
Median of Cr (μg/g)		0.23	0.58	1.23	3.49	
Adjusted OR (95% CI)		1 (ref.)	0.90(0.28-2.93)	0.60(0.17-2.04)	0.39(0.09-1.74)	0.197
Adjusted OR[5] (95% CI)		1 (ref.)	0.79(0.22-2.85)	0.55(0.14-2.18)	0.30(0.06-1.50)	0.126
Adjusted OR[6] (95% CI)		1 (ref.)	0.87(0.23-3.35)	0.60(0.14-2.66)	0.24(0.04-1.48)	0.114

[1]Quartiles based on distribution of toenail chromium among controls. [2] Adjusted for sex, age (continuous) and PREDIMED center (indicator variables). [3]Additionally adjusted for smoking (binary), hypertension, hypercholesterolemia, diabetes, family history of premature coronary heart disease, body mass index (continuous) and alcohol intake (three categories: <5/10 g/day, 5-25/10-50 g/day, >25/50 g/day in women/men). [4]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). [5]Additionally adjusted for smoking (binary), hypertension, hypercholesterolemia, family history of premature coronary heart disease, body mass index (continuous) and alcohol intake (three categories: <5/10 g/day, 5-25/10-50 g/day, >25/50 g/day in women/men). [6]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).

Supplemental Table 2. Center-Adjusted and multivariable odds ratios for stroke by quartile of baseline toenail chromium level among cases and controls, the PREDIMED trial.

Variable		Quartiles[1] of toenail chromium				
	number of cases/controls	Q1	Q2	Q3	Q4	p for trend
Total sample[2]						
Cases / controls	86/271	32/68	16/68	24/68	14/67	
Median of Cr (μg/g)		0.19	0.45	1.07	2.96	
Adjusted OR (95% CI)		1 (ref.)	0.53(0.26-1.10)	0.77(0.40-1.51)	0.49(0.23-1.06)	0.179
Adjusted OR[3] (95% CI)		1 (ref.)	0.55(0.26-1.16)	0.83(0.41-1.66)	0.48(0.22-1.06)	0.163
Adjusted OR[4] (95% CI)		1 (ref.)	0.48(0.23-1.03)	0.70(0.34-1.45)	0.52(0.23-1.19)	0.307
Type 2 diabetes at baseline[2]						
Cases / controls	51/133	14/33	11/34	12/33	14/33	
Median of Cr (μg/g)		0.14	0.36	0.84	2.61	
Adjusted OR (95% CI)		1 (ref.)	0.77(0.29-2.06)	0.78(0.28-2.15)	1.07(0.40-2.87)	0.667
Adjusted OR[5] (95% CI)		1 (ref.)	0.65(0.22-1.90)	0.74(0.25-2.19)	1.09(0.39-3.04)	0.569
Adjusted OR[6] (95% CI)		1 (ref.)	0.50(0.16-1.58)	0.70(0.22-2.27)	1.29(0.42-3.99)	0.325
No type 2 diabetes at baseline[2]						
Cases / controls	35/138	14/35	6/34	12/35	3/34	
Median of Cr (μg/g)		0.23	0.58	1.23	3.49	
Adjusted OR (95% CI)		1 (ref.)	0.48(0.15-1.54)	0.87(0.32-2.40)	0.22(0.05-0.92)	0.067
Adjusted OR[5] (95% CI)		1 (ref.)	0.45(0.13-1.51)	0.92(0.32-2.67)	0.20(0.05-0.87)	0.055
Adjusted OR[6] (95% CI)		1 (ref.)	0.46(0.13-1.60)	0.76(0.24-2.43)	0.22(0.05-1.00)	0.078

[1]Quartiles based on distribution of toenail chromium among controls. [2] Adjusted for sex, age (continuous) and PREDIMED center (indicator variables). [3]Additionally adjusted for smoking (binary), hypertension, hypercholesterolemia, diabetes, family history of premature coronary heart disease, body mass index (continuous) and alcohol intake (three categories: <5/10 g/day, 5-25/10-50 g/day, >25/50 g/day in women/men). [4]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). [5]Additionally adjusted for smoking (binary), hypertension, hypercholesterolemia, family history of premature coronary heart disease, body mass index (continuous) and alcohol intake (three categories: <5/10 g/day, 5-25/10-50 g/day, >25/50 g/day in women/men). [6]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).