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Myocardial infarction risk in relation to zinc concentration in toenails

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Zn is an essential mineral. The role of Zn in atherosclerosis is not clear. Epidemiological studies, which have reported contradictory results, are limited by the use of serum Zn levels as a marker of intake. We assessed the association of toenail Zn, which integrates dietary Zn intake over 3 to 12 months, with the risk of a first myocardial infarction. Toenail Zn concentrations were determined by neutron activation analysis in the European multi-centre case–control study on antioxidants, myocardial infarction and breast cancer. This multi-centre case–control study included 684 cases and 724 controls from eight European countries and Israel. Toenail Zn levels of controls (adjusted for age and study centre) were positively associated with age, α-tocopherol and Se, but not with additional dietary variables or with classical risk factors for CHD. Average toenail Zn was 106·0 mg/kg in cases (95 % CI 103·1, 108·9) and 107·5 mg/kg in controls (95 % CI 104·5, 110·7). After controlling for cardiovascular risk factors and for centre, the adjusted odds ratios of myocardial infarction for quintiles 2–5 of toenail Zn with respect to the first quintile were 0·97 (95 % CI 0·59, 1·58), 1·15 (95 % CI 0·72, 1·85), 0·91 (95 % CI 0·56, 1·50), and 0·85 (95 % CI 0·52, 1·39). The *P* for trend was 0·45. In conclusion toenail Zn levels (reflecting long-term dietary intake) were not significantly associated with acute myocardial infarction.

Case–control studies: Heavy metals: Zinc: Myocardial infarction: Neutron activation analysis

Zn is an essential mineral necessary for maintenance of membrane structure and function. There are over 300 known enzymes that require Zn for catalytic, co-catalytic or structural functions (Prasad, 1993). Clinical manifestations of Zn deficiency include growth retardation, male hypogonadism, skin changes, mental lethargy and susceptibility to infection. Zn deficiency also results in increased membrane cholesterol and altered cell membrane skeleton protein composition (Bettger & O'Dell, 1981; King, 1990; Vallee & Falchuk, 1993; Chesters, 1997).

The effect of Zn in the pathogenesis of atherosclerosis is not clear. Epidemiological studies have reported contradictory results and are limited by the use of serum Zn levels as a marker of Zn intake. Nevertheless, an anti-atherogenic effect of Zn has been hypothesized due to its antioxidant and membrane-stabilizing properties (Henning *et al.* 1996, 1999).

To evaluate the association of Zn with the risk of myocardial infarction, we measured toenail Zn in the participants in the European multi-centre case–control study on

Abbreviations: EURAMIC, European multi-centre case–control study on antioxidants, myocardial infarction and breast cancer; PL, project leader; PMG, project management group.

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antioxidants, myocardial infarction and breast cancer (EURAMIC) (Kardinaal *et al.* 1993, 1997) and assessed the association with the risk of a first acute myocardial infarction.

Methods

Design and subjects

The target population of the EURAMIC study consisted of men 70 years of age or younger, native residents of Finland (Helsinki), Germany (Berlin), Israel (Jerusalem), The Netherlands (Zeist), Norway (Sarpsborg), Russia (Moscow), UK (Edinburgh, Scotland), Switzerland (Zürich), and Spain (Granada and Málaga). Subjects were excluded if they had a previous diagnosis of myocardial infarction, drug or alcohol abuse, major psychiatric disorders, if they were institutionalized, or had modified their dietary pattern in the past year (Kardinaal *et al.* 1993).

Cases were men with a first acute myocardial infarction, confirmed by characteristic electrocardiographic changes and serum enzymes, hospitalized within 24 h from the onset of symptoms. They were recruited from the coronary care units of participating hospitals. Controls were men without a history of myocardial infarction, recruited from the study population catchment area and frequency-matched for age in 5-year intervals. In Finland, Israel, Germany, Scotland and Switzerland, random sampling from local population registers was used for control selection. In Russia and in the two Spanish centres, controls were selected from patients admitted to the hospital for disorders not known to be associated with dietary factors (renal colic, non-infectious prostatism, acute appendicitis, non-infectious ear disease, hernia, volvulus, rectal or anal disease except cancer, haemorrhoids, or chronic infection). In The Netherlands, controls were selected from the catchment area of the patient's general practitioner, and in Norway they were selected by inviting friends and relatives of the case.

Cases and controls were recruited concurrently during 1991 and 1992. Informed consent was obtained from study participants in accordance with the ethical standards of the responsible local committees on human experimentation.

Data collection

Information on smoking habits, history of hypertension, and diabetes was collected for all subjects by standard questionnaires (Rose *et al.* 1982). Socio-economic status, family history and alcohol intake were assessed through locally developed questionnaires by administered interview.

Toenail clippings from all ten toes were collected within 8 weeks of inclusion in the study and were stored in small plastic bags at room temperature (Kardinaal *et al.* 1997). The mean weight of the samples was 53·9 (SD 39·1) mg.

A non-fasting venous blood sample was drawn for cholesterol analysis. In cases, these samples were drawn within 24 h of hospital admission or onset of symptoms. Serum samples were transported on dry ice at -56°C to the reference laboratories.

Subcutaneous adipose tissue was taken from the buttock, by needle aspiration, within 7 d of hospital admission. Samples were stored at -70°C and were analysed in a central laboratory.

Laboratory analyses

The Zn concentration in toenails was measured by instrumental neutron activation analysis at the Interfaculty Reactor Institute of the Delft University of Technology in Delft, The Netherlands (Alfassi, 1994; Bode & de Goeij, 1998; Bode, 2000). Toenail clippings were irradiated for 4 h in a thermal flux of 5×10^{12} neutrons/s per cm^2. After a decay time of 21 d, the γ radiation of Zn was measured in a well-type Ge-detector. For each centre, samples from cases and controls were analysed together and randomly distributed across batches. Personnel at the Interfaculty Reactor Institute were blinded with respect to the case or control status of the samples.

The CV, as an expression of inter-assay variation for Zn, was 3·2 % for fifty samples of reference material NIST SRM-1577b 'Bovine Liver' (NIST, Gaithersburg, MD, USA) with a certified value of 127 ± 16 (1 SD) mg/kg and also for fifty-two samples of reference material BCR-CRM-414 'Trace elements in plankton' (IRMM, Retieseweg, Belgium) with a certified value of 112 ± 3 (1 SD) mg/kg.

Serum total cholesterol levels were determined enzymically (Boehringer-Mannheim GmbH, Mannheim, Germany). HDL-cholesterol was determined after precipitation with dextran sulfate and $MgCl_2$. Cholesterol determinations were performed at the National Public Health Institute in Helsinki, Finland.

α-Tocopherol and β-carotene were determined in the adipose tissue after saponification by reverse-phase HPLC and spectrophotometric detection (Van Vliet *et al.* 1991).

Statistical methods

Since the distributions of Zn in all centres were right-skewed, log-transformations were used. The results are reported as geometric means (mg/kg). The distribution of Zn in controls was used to compute cut-off points and medians for quintiles of exposure. The levels of cardiovascular risk factors across quintiles of Zn were compared among controls by ANOVA and χ^2 tests (Snedecor & Cochran, 1989). The centre-specific and adjusted overall mean case–control differences of Zn and 95 % CI were estimated by linear regression (Hosmer & Lemeshow, 2000).

For multivariate analysis, the association of Zn with the risk of myocardial infarction was estimated with multiple logistic regression. Adjusted relative risks were estimated as odds ratios in quintiles 2–5 using the lowest quintile as the reference category, and trend tests across quintiles of Zn were computed by including in the logistic models an ordinal variable with the median for each quintile of Zn. All *P* values reported were two-tailed. Statistical analyses were performed using STATA (StataCorp, 1999) and the SAS package (SAS Institute, 1990).

Results

Compared with controls, cases had significantly higher BMI, lower HDL-cholesterol levels, and were more

likely to be hypertensive, diabetic, smokers, or to have a family history of CHD (Table 1). We believe that the lower concentration of total cholesterol among cases most probably reflects the effect of acute myocardial infarction. Therefore total cholesterol was not further considered in case–control comparisons.

Average toenail Zn was 106·0 mg/kg in cases (95 % CI 103·1, 108·9) and 107·5 mg/kg in controls (95 % CI 104·5, 110·7). The relative combined uncertainty in the individual measurements varied between 2 to 4 %, whereas the limit of detection for Zn in toenail samples was estimated at approximately 5 mg/kg.

Zinc levels and risk factors among controls

Jerusalem and Zürich had the highest average concentrations of Zn (geometric means of 128·1 and 115·3 mg/kg, respectively), while Zeist, Sarpsborg and Helsinki had the lowest (geometric means of 101·6, 102·5 and 102·8 mg/kg, respectively). The maximum difference between centres was only 27 % (Table 2). The age- and centre-adjusted overall geometric mean of Zn in controls was 107·5 mg/kg (95 % CI 104·5, 110·7). The distribution of Zn in each centre and across centres was relatively narrow, with a CV of 8·4 % for the total of the control sample, and below 10 % in all centres except in the case of Jerusalem.

Table 3 shows the association of toenail Zn levels with other coronary risk factors among controls, adjusted for age and centre. Zn was associated with age, α-tocopherol and Se, whereas no significant association was found in relation to other factors (BMI, total cholesterol, HDL-cholesterol, alcohol, β-carotene, history of hypertension, smoking, diabetes, socio-economic status or family history of CHD).

Toenail zinc and risk of myocardial infarction

Zn levels were lower in cases compared with controls in most centres (Table 2), but the difference was small and statistically non-significant (except in Jerusalem). There was no significant effect modification by centre on the association of Zn with myocardial infarction. After adjusting for age and centre, BMI, HDL-cholesterol, smoking,

Table 1. Comparison of cardiovascular risk factors between cases and controls
(Mean values and standard deviations)

	Cases (*n* 684)		Controls (*n* 724)		
Risk factor	Mean	SD	Mean	SD	*P* value
Age (years)	54·7	8·9	53·2	9·3	0·002
BMI (kg/m^2)	26·5	3·9	25·9	3·4	0·004
Total cholesterol (mmol/l)	5·5	1·1	5·6	1·1	0·110
HDL-cholesterol (mmol/l)	0·9	0·3	1·1	0·3	<0·001
History of hypertension (%)	26·0		17·4		<0·001
Smoking (% current smokers)	61·4		37·5		<0·001
Diabetes mellitus (%)	8·4		3·9		<0·001
Alcohol use (g/d)	18·2		17·8		0·750
Family history of CHD (%)	57·6		45·3		<0·001
Low socio-economic status (%)	25·1		20·8		0·036

Table 2. Zinc levels and case–control ratios of zinc levels (mg/kg) in toenails
(Mean values and 95 % confidence intervals)

	n		Cases		Controls		Zn case:Zn control	
Centre	Cases	Controls	Mean	95 % CI	Mean	95 % CI	Ratio	95 % CI
Berlin	75	97	104·6	98·1, 111·5	108·3	101·1, 116·1	0·95	0·86, 1·05
Edinburgh	39	25	103·7	96·1, 111·9	112·9	101·4, 125·7	0·92	0·81, 1·05
Granada	55	52	109·4	95·7, 125·0	110·1	97·8, 124·1	0·99	0·83, 1·18
Helsinki	56	62	102·7	98·6, 107·0	102·8	98·6, 107·1	1·00	0·95, 1·06
Jerusalem	57	59	103·1	95·8, 111·0	128·1	105·7, 155·3	0·80	0·65, 0·99
Málaga	94	100	110·2	100·2, 121·3	102·9	96·5, 109·7	1·07	0·96, 1·20
Moscow	92	97	110·5	99·2, 123·1	103·8	95·4, 113·0	1·07	0·92, 1·23
Sarpsborg	96	101	101·8	98·0, 105·9	102·5	98·8, 106·5	0·99	0·94, 1·05
Zeist	64	57	99·3	94·0, 104·9	101·6	95·3, 108·5	0·98	0·90, 1·06
Zürich	56	74	113·8	100·0, 129·5	115·3	102·4, 130·1	0·96	0·80, 1·15
Overall	684	724	106·0	103·1, 108·9	107·5	104·5, 110·7	0·99*	0·95, 1·03
							0·96†	0·91, 1·01
							0·96‡	0·92, 1·01

* Age- and centre-adjusted.
† Further adjusted by BMI, HDL-cholesterol, history of hypertension, smoking, alcohol intake, diabetes, family history of CHD, α-tocopherol, β-carotene and Se.
‡ Further adjusted by socio-economic status.

Table 3. Age- and centre-adjusted levels of risk factors by quintiles (Q) of zinc among controls*

Risk factor	Q1	Q2	Q3	Q4	Q5	*P* for trend
Age (years)	53·1	51·8	53·0	54·0	55·3	0·006
BMI (kg/m^2)	25·6	25·8	25·6	26·4	26·0	0·16
Total cholesterol (mmol/l)	5·4	5·6	5·5	5·5	5·7	0·09
HDL-cholesterol (mmol/l)	1·07	1·12	1·11	1·06	1·11	0·69
Alcohol (g/week)	124·8	134·8	129·5	109·9	142·1	0·66
α-Tocopherol† (μg/g)	188·3	175·2	188·7	185·4	215·7	0·03
β-Carotene† (μg/g)	0·43	0·46	0·44	0·44	0·43	0·73
Se (mg/kg)	0·58	0·61	0·63	0·64	0·68	0·0001
History of hypertension (%)	12·6	14·9	13·0	9·3	12·8	0·67
Smoking (% current smokers)	41·0	44·5	35·3	35·3	36·3	0·19
Diabetes mellitus (%)	2·4	2·5	1·8	3·3	1·8	0·74
Family history of CHD (%)	43·5	41·9	46·8	46·2	41·3	0·76
Low socio-economic status (%)	16·6	18·2	12·8	14·5	17·1	0·96

* Cut-off values for quintiles of Zn in controls were 88·4, 96·8, 106·5 and 118·9 mg/kg (the mean value of each quintile was 82·5, 93·1, 101·5, 111·9 and 134·6 mg/kg).
† Geometric means.

alcohol drinking, history of hypertension, diabetes and family history of CHD, cases had lower concentrations of Zn by 4 % (95 % CI 9, −1). These estimations were further adjusted by α-tocopherol, β-carotene and Se, and then by socio-economic status, but controlling for these variables did not materially affect the estimates.

The association of increased Zn with lower risk of myocardial infarction was examined in more detail by evaluating the odds ratios of disease by quintiles of Zn (Table 4). Age- and centre-adjusted analysis showed a 19 % reduction in the risk of myocardial infarction for the fifth quintile, but no significant trend was found ($P=0·25$). Significant interactions between Zn and other trace elements or fatty acids were not detected (data not shown). Further adjustments for BMI, HDL-cholesterol, smoking, alcohol drinking, history of hypertension, diabetes, family history of CHD, α-tocopherol, β-carotene and Se, and then by socio-economic status, tended to slightly move the estimates towards the null value.

Discussion

In this large, international case–control study, the concentration of Zn in the toenails of study participants was slightly lower among cases of myocardial infarction compared with controls. However, this difference was not statistically significant.

There are some factors that should be pointed out for the appropriate appraisal of our findings. First, the study was specifically designed to evaluate the association of toenail heavy metals with the risk of myocardial infarction. Second, toenails and adipose tissue samples were originally collected in cases shortly after the myocardial infarction. These measurements are thus unlikely to be affected by the manifestation of disease, a common limitation of retrospective case–control studies. Finally, in contrast to heavy metal levels in serum or plasma, which may show substantial day-to-day variability, toenail clippings provide a measure of relatively long-term intake of trace elements, reflecting average exposure to heavy metals over the previous 3–12 months (Hunter, 1998). Moreover, there is evidence of particularly good reproducibility of toenail levels of Zn over a long period of time (Garland *et al.* 1993).

Reviewing the main physiological aspects of this heavy metal, it is important to mention that Zn functions as a component of various enzymes that depend on Zn for catalytic activity (Fabris & Mocchegiani, 1995). Another biological function of Zn is the maintenance of the integrity of proteins

Table 4. Risk of first myocardial infarction by quintiles (Q) of zinc (Relative risk ratios and 95 % confidence intervals)

	Q1	Q2	Q3	Q4	Q5	*P* for trend
Cases (*n*)	153	124	152	132	123	
Controls (*n*)	145	145	146	143	145	
Median (mg/kg)	82·5	93·1	101·5	111·9	134·6	
Model 1*	1	0·85	1·00	0·87	0·81	0·25
95 % CI		0·61, 1·18	0·73, 1·39	0·63, 1·22	0·58, 1·13	
Model 2†	1	0·97	1·15	0·91	0·85	0·45
95 % CI		0·59, 1·58	0·72, 1·85	0·56, 1·50	0·52, 1·39	
Model 3‡	1	0·99	1·17	0·95	0·93	0·69
95 % CI		0·60, 1·63	0·73, 1·90	0·58, 1·57	0·56, 1·53	

* Age- and centre-adjusted.
† Further adjusted by BMI, HDL-cholesterol, history of hypertension, smoking, alcohol intake, diabetes, family history of CHD, α-tocopherol, β-carotene and Se.
‡ Further adjusted by socio-economic status.

required for the stability of membrane structures (Henning *et al.* 1999). Finally, Zn has a role as a regulator of gene expression (Tamura *et al.* 1996; Archer *et al.* 2001). While knowledge of the biochemical and molecular genetics of Zn function is well developed and expanding, no relationship of these genes to Zn deficiency, toxicity or functions for which Zn is particularly critical have been clearly established.

Zn is widely distributed in food, but the majority of Zn is present as free ion. Therefore, its bioavailability seems to be a function of the extent of absorption and digestion (Prasad, 1993; Vallee & Falchuk, 1993). Almost all Zn is absorbed by the small intestine through a transcellular process. Considerable amounts of Zn come from endogenous sources. Zn depletion in man is accompanied by reduced endogenous Zn loss and an increase in the efficiency of intestinal Zn absorption. Regulation of absorption may provide a 'coarse control' of body Zn, whereas endogenous Zn release provides 'fine control' to maintain balance. In this context, circulating Zn in plasma provides an insensitive index of Zn status. Moreover, plasma Zn levels are strongly affected by acute-phase stimuli, such as acute myocardial infarction (Craig *et al.* 1990; Pucheu *et al.* 1995). Therefore, it is far more appropriate to use toenail Zn as a biomarker, since it has been shown to be a reliable indicator of long-term Zn intake.

In the present study, we found up to 27 % difference in the Zn levels among controls studied across centres. This relatively low level of variation suggests that toenail Zn concentrations could be affected by the homeostatic mechanisms (and, therefore, relatively modest differences in Zn intake would not be easily detected), or that this particular study population (although arising from different European centres) shows little variability in Zn intake.

Regarding the anti-atherogenic effect of Zn, an antioxidant and membrane-stabilizing effect has been hypothesized (Henning, 1996, 1999). Experimental studies have shown that Zn protects against metabolic physiological derangements of the vascular endothelium and interferes with signalling pathways involved in apoptosis, whereas Zn deficiency causes an impairment of endothelial barrier function. The results of epidemiological studies are inconsistent, although most of them found lower levels of serum Zn levels in patients with differing manifestations of cardiovascular disease than in apparently healthy controls (Manthey *et al.* 1981; Khan *et al.* 1984; Marniemi *et al.* 1988; Oster *et al.* 1989; Iskra *et al.* 1993; Martin-Lagos *et al.* 1997). However, the serum levels of Zn measured in these study designs (basically case–control studies) were analysed after the occurrence of the cardiovascular event, where a decrease of Zn levels is probably a non-specific secondary manifestation of the acute-phase reaction (Craig *et al.* 1990; Pucheu *et al.* 1995).

The main epidemiological studies where the measurement of serum Zn levels was carried out before the occurrence of the cardiovascular event were two nested case–control studies. Both were well designed although had somewhat different conclusions. The first reported no significant change in the risk of death from cardiovascular disease, although the authors pointed out that the data were compatible with a protective effect of a high Zn level (Kok *et al.* 1988). The second showed that lower serum Zn was an independent predictor of cardiovascular death, with a relative risk of 0·57 (95 % CI 0·35, 0·93) in the highest third of serum Zn levels (Reunanen *et al.* 1996).

In conclusion, our results in this multi-centre study population suggest that toenail Zn levels, which reflect integration of dietary Zn intake and exposure over time, are not significantly associated with acute myocardial infarction. It is possible that the relative homogeneity of Zn levels and probably also intake in these populations does not permit detection of an association. Nevertheless, these results do not preclude the existence of an association in populations with less homogeneous Zn levels. For example, the Jerusalem centre, in which there was a greater variation in intake, showed a significant within-population protective association of higher Zn levels. Therefore, we believe that studies in populations with greater variability in Zn levels would be of substantial interest.

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