Review

Toenail Trace Element Levels as Biomarkers: Reproducibility over a 6-Year Period[1]

Miriam Garland, J. Steven Morris, Bernard A. Rosner, Meir J. Stampfer, Victoria L. Spate, Connie J. Baskett, Walter C. Willett, and David J. Hunter[2]

Departments of Epidemiology [M. G., M. J. S., D. J. H., W. C. W.] and Nutrition [W. C. W.], Harvard School of Public Health, Boston, Massachusetts 02115; Research Reactor Facility, University of Missouri, Columbia, Missouri 65211 [J. S. M., V. L. S., C. J. B.]; and Channing Laboratory, Department of Medicine, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115 [B. A. R., M. J. S., W. C. W., D. J. H.]

Abstract

We assessed the reproducibility over a 6-year period of 16 trace elements measured in toenails by comparing levels in paired specimens collected in 1982–1983 and 1988 from 127 women in the United States. The Spearman correlation coefficients for the reproducibility of toenail levels of selenium and arsenic (both known to reflect intake of these elements) were 0.48 and 0.54. Correlations for other elements ranged from 0.26 (copper) to 0.58 (zinc). In utilizing biomarkers to assess exposure in epidemiological studies of cancer and other chronic diseases, random within-person variability in exposure leads to attenuation of measures of association between exposure and disease. We demonstrate the effect of such variability on odds ratios from a hypothetical case-control study. For a true odds ratio of 3.0 (for a comparison of the highest quintile *versus* the remaining 4 quintiles of exposure) the odds ratios which would be observed in the presence of the degree of within-person variability demonstrated in this study were 2.15 for toenail arsenic and 1.67 for toenail copper levels. Toenail concentrations of certain trace elements are useful biomarkers of exposure in which a single sample is assumed to represent long-term exposure. However, substantial attenuation in measures of association may occur.

Introduction

There is growing interest in the relationship between intake of certain trace elements and cancer. Selenium, for instance, inhibits carcinogenesis in many animal models, and inverse associations between selenium intake and both total and site-specific cancer mortality have been reported in ecological studies (1). Occupational exposure to arsenic has been shown to increase lung cancer risk, and oral intake of arsenic appears to increase risk of nonmelanoma skin cancer and possibly risk of cancer at other sites (2). High body iron stores may increase cancer risk (3).

Biomarkers of exposure to trace elements are potentially useful in epidemiological studies both as a measure of intake and as a means to validate other forms of exposure assessment (4). Measuring levels of trace elements in toenail clippings is a promising method, since these clippings are more convenient to collect and store than blood and are less subject to external contamination than hair or fingernails (5, 6). Because the induction period for most cancers is thought to be many years or even decades, it is usually desirable that a biochemical marker reflect long-term exposure. In contrast to trace element levels in serum, plasma, or whole blood, which may vary substantially from day to day, toenail clippings may provide a measure of relatively long-term trace element intake. Each clipping represents several weeks of growth, and, as nails from different toes vary in the time between formation and clipping, nails from all 10 toes are likely to reflect exposure integrated over the previous 3–12 months and possibly longer (7).

If a biomarker has been shown to be responsive to intake, then an assessment of its long-term reproducibility is desirable to assess how well a single measurement reflects long-term exposure. We therefore assessed the reproducibility of measurements of 16 trace elements in toenails by comparing levels in specimens from 127 U.S. women collected in 1982–1983 and again in 1988. As random within-person variability of exposure measurements in an epidemiological study attenuates estimates of association based on a single measurement, we demonstrate the impact of such variability on estimates of the odds ratio in a hypothetical case-control study.

If no data are available on the validity of elemental nail levels as an indicator of intake, an initial assessment of potential utility can be made by determining reproducibility of the nail element levels. As an example of an assessment of the validity of using nail levels of an element as a measure of intake, we assessed the association between fish intake (the major source of dietary mercury) and toenail mercury levels.

Methods

Population. In 1976, 121,700 female registered nurses aged 30–55 years living in 11 U.S. states completed a mailed questionnaire including items on risk factors for cancer and coronary heart disease (8). Every 2 years, participants respond to follow-up questionnaires to update information on potential risk factors and to identify new cases of disease. In December 1982, participants were asked to provide a set of

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[2] To whom requests for reprints should be addressed, at Channing Laboratory, 180 Longwood Avenue, Boston, MA 02115.

Table 1 Nuclear parameters for the elements measured via instrumental neutron activation analysis

Target nuclide (Experiment)	Isotopic abundance	Product nuclide	Half-life	γ-ray energy (KeV)	Sensitivity (μg/g)[a]
Al-27 (2)	1.0	Al-28	2.24 m[b]	1779	0.08
As-75 (3)	1.00	As-76	26.32 h	559.1	0.005
Br-81 (3)	0.4931	Br-82	35.30 h	776.5	0.005
Ca-46 (3)	0.00004	Ca-47	4.54 d	1297	40
Cl-37 (2)	0.2423	Cl-38	37.24 m	1642.2	4
Cr-50 (4)	0.0435	Cr-51	27.70 d	320.1	0.04
Co-59 (4)	1.00	Co-60	5.27 y	1332.5	0.02
Cu-65 (2)	0.3083	Cu-66	5.10 m	1039.2	0.4
Fe-58 (4)	0.0028	Fe-59	44.50 d	1099.2	4
Mg-26 (2)	0.1101	Mg-27	9.46 m	1014.4	8
Hg-202 (4)	0.2980	Hg-203	46.61 d	279.2	0.04
Sc-45 (4)	1.00	Sc-46	83.81 d	889.3	0.0004
Se-76 (1)	0.0900	Se-77m	17.4 s	161.9	0.01
S-36 (2)	0.0002	S-37	5.05 m	3104	800
Ti-50 (2)	0.0540	Ti-51	5.76 m	320.1	0.4
Zn-64 (4)	0.4860	Zn-65	243.9 d	1115.6	0.2

[a] Detection limit assuming a 25-mg sample.
[b] d, days; h, hours; m, minutes; s, seconds; y, years.

nail clippings from all 10 toes to be used in nested case-control studies of cancer and diabetes, and 68,213 did so. Almost all specimens arrived in 1982 or 1983. The 62,641 women who had no prior history of cancer (other than nonmelanoma skin cancer) constituted the baseline cohort. In a nested case-control study of selenium levels in nails in relation to breast cancer risk, 434 cases of breast cancer were identified; for each case a control was chosen matched on year of birth and calendar month of return of the nail specimens (8). Of these controls, 150 were selected at random and asked to return a second set of toenail clippings in 1988. Of these, 128 women responded. One sample was lost, leaving 127 women for analysis. The median time between return of the first and second sets of toenail clippings was 68 months.

Laboratory Analyses. Concentrations of 16 elements were determined by instrumental neutron activation analysis. The measurements were made at the University of Missouri Research Reactor located in Columbia, MO. Stable target nuclides, their isotopic abundances, product nuclides, and half-lives are given in Table 1. The photon energies for the γ-rays used to quantify the elements are also shown, along with the detection limits.

The 127 paired samples were analyzed in the same laboratory run with staff blinded to pair status. All samples were first cleaned with deionized water using a sonicator and then weighed into precleaned high-density polyethylene vials for irradiation. Whenever possible, a sample size of at least 25 mg was used. Each cleaned sample was subjected to four instrumental neutron activation analysis experiments done in serial fashion. Standard comparison was used in Experiment 1 (to determine selenium), and the k_0 method (9, 10) was adapted for Experiments 2 (to determine aluminum, chlorine, copper, magnesium, sulfur, and titanium), 3 (to determine arsenic, bromine, and calcium), and 4 (to determine chromium, cobalt, iron, mercury, scandium, and zinc). The neutron spectrum for Experiments 1 and 2 included thermal (ϕ_{th}) and epithermal (ϕ_{epi}) flux components of, respectively:

$$\phi_{th} = 8 \times 10^{13} n \cdot cm^{-2} \cdot sec^{-1}$$

and

$$\phi_{epi} = 2 \times 10^{12} n \cdot cm^{-2} \cdot sec^{-1}$$

The neutron flux components for Experiments 3 and 4 were reduced by approximately 50%. High resolution γ ray spectroscopy was used in all four experiments to measure the induced radioactivity.

Selenium was determined in Experiment 1 by measuring the Se-77m radionuclide produced during a 5-s irradiation and detected following a 15-s decay using a 25-s counting period. In experiment 2, concentrations of aluminum, chlorine, copper, magnesium, sulfur, and titanium were determined from the activities of Al-28, Cl-38, Cu-66, Mg-27, S-37, and Tl-51, respectively, which were produced during a 1-min irradiation and detected during a 10-min count following a 5-min decay period. An irradiation period of approximately 50 h was used in Experiments 3 and 4. In Experiment 3, the concentrations of arsenic, bromine, and calcium were measured by a 2-h counting period following a 2–5-day decay using As-76, Br-82, and Ca-47, respectively. Finally, in Experiment 4, chromium, cobalt, iron, mercury, scandium, and zinc concentrations were determined from Cr-51, Co-60, Fe-59, Hg-203, Sc-46, and Zn-65, respectively, using a second counting period of 4–6 h following several weeks of additional decay.

Element levels were adjusted for the weight of the specimen by regressing the log of the toenail element values (the dependent variable) against the nail weight (the independent variable), adding the residual of each observation to the predicted mean toenail value, and exponentiating (11).

Data Analyses. For certain elements (arsenic, calcium, copper, magnesium, and titanium) the values observed for some toenail specimens were below the detection limit value (the sensitivity of the analytic technique). For these elements, estimates of the mean and variance were derived by treating values below the detection limit value as left-censored observations and utilizing failure-time techniques based on a log-normal distribution of the data (12) using the SAS Lifereg procedure (13). Observations which were below the detection limit value were assigned their expected value, which

Table 2 Mean and median values (μg/g) for trace element levels among 127 U.S. women in 1982–1983 and 1988, and Spearman's correlation coefficient for reproducibility over this 6-year period

Element	(yr)	n[a]	Mean (SD) (μg/g)	Median (μg/g)	Within-to-between person variance ratio	Spearman's r[b]
Aluminum	(1982)	0	14.2 (10.9)	10.9	1.80	0.39
	(1988)	0	14.4 (18.6)	9.72		
Arsenic	(1982)	7	0.11 (0.17)	0.083	1.01	0.54
	(1988)	12	0.12 (0.27)	0.074		
Bromine	(1982)	0	2.54 (1.72)	2.18	2.49	0.35
	(1988)	0	2.41 (1.22)	2.06		
Calcium	(1982)	32	827 (403)	747[c]	1.67	0.44
	(1988)	17	968 (436)	901***		
Chlorine	(1982)	0	830 (409)	730	1.59	0.39
	(1988)	0	782 (440)	677		
Chromium	(1982)	0	2.43 (3.16)	1.80	2.27	0.33
	(1988)	0	2.39 (2.91)	1.75		
Cobalt	(1982)	0	0.044 (0.034)	0.036	1.85	0.35
	(1988)	0	0.042 (0.023)	0.035		
Copper	(1982)	5	5.21 (3.34)	4.51	3.20	0.26
	(1988)	6	4.33 (2.29)	3.84**		
Iron	(1982)	0	46.9 (35.3)	39.6	1.44	0.43
	(1988)	0	42.5 (26.3)	36.9*		
Magnesium	(1982)	1	167 (130)	135	1.03	0.46
	(1988)	0	172 (80.1)	149		
Mercury	(1982)	0	0.87 (5.57)	0.26	0.58	0.56
	(1988)	0	0.67 (3.81)	0.26		
Scandium	(1982)	0	0.0054 (0.0042)	0.0042	1.17	0.48
	(1988)	0	0.0053 (0.0062)	0.0035		
Selenium	(1982)	0	0.83 (0.16)	0.81	1.85	0.48
	(1988)	0	0.92 (0.15)	0.89***		
Sulfur	(1982)	0	28218 (2600)	28618	0.95	0.48
	(1988)	0	27376 (3132)	27400***		
Titanium	(1982)	31	12.0 (15.3)	6.87	1.92	0.33
	(1988)	34	12.8 (25.7)	6.11		
Zinc	(1982)	0	115 (39.1)	106	1.32	0.58
	(1988)	0	110 (28.9)	105*		

[a] Number of observations below the limit of detection.
[b] Spearman's correlation coefficient for reproducibility over the period 1982–1983 to 1988; $P < 0.003$ for all elements ($P = 0.05$ for $r = 0.17$; $P = 0.01$ for $r = 0.22$; $P = 0.001$ for $r = 0.28$).
[c] *** $P \leq 0.001$; * $P \leq 0.05$; ** $P \leq 0.01$; (from the Wilcoxon signed rank test for differences in trace element levels between 1982 and 1988).

can be calculated based on the mean and variance of the distribution of the $\log_e$-transformed trace element (derived by the failure-time procedure described above) and the detection limit value (see "Appendix").

The Wilcoxon signed rank test (14) was used to evaluate differences in toenail levels of the trace elements between 1982–1983 and 1988.

Reproducibility was assessed by comparing the 1982–1983 toenail trace element levels of each participant with her 1988 levels of the corresponding element, using Spearman correlation coefficients. Estimates of within- and between-person components of variance were calculated from a random effects analysis of variance model using $\log_e$-transformed values. In the case of arsenic, these variance components were estimated after the exclusion of two extreme outliers. For two elements, copper and arsenic, the impact of random within-person variability of an exposure measure on odds ratios from a hypothetical case-control study was demonstrated using the method given by Willett (11).

Frequency of fish consumption was derived from a single question on the 1982 questionnaire and its correlation with 1982–1983 toenail mercury and arsenic levels were examined. The 1986 questionnaire included four separate questions on the frequency of consumption of specific types of fish, and the correlation of each of these four categories of fish intake with 1988 toenail mercury and arsenic levels was determined. In addition, the frequencies of 1986 intake of the four specific types of fish were combined to derive a frequency of total fish consumption; the correlation of 1986 total fish consumption with toenail mercury and arsenic levels was examined.

Results

The mean and median values (in μg/g) for toenail trace element levels for 1982–1983 and for 1988 are shown in Table 2. Sulfur was the predominant element (sulfur is the element responsible for cross-linking keratin in nails). Calcium and chlorine were measured at a concentration of approximately 1 mg/g, with zinc and magnesium measured between 0.1 and 0.2 mg/g. All other elements were less than 100 μg/g, with cobalt and scandium concentrations of less than 0.1 μg/g. Calcium and selenium levels increased significantly over the study period, while copper, iron, sulfur, and zinc levels decreased significantly.

The Spearman correlation coefficients for the reproducibility of the toenail trace element levels over 6 years are shown in Table 2. All elements examined were significantly positively correlated over time; the values of these

Table 3 Observed odds ratios from a hypothetical case-control study[a] caused by attenuation due to random within-person variability in the exposure measure

Exposure	True OR, 3.0[b]	True OR, 1.5
Toenail arsenic as exposure measure (w/b[c] = 1.01)	2.15	1.32
Toenail copper as exposure measure (w/b = 3.20)	1.67	1.21

[a] The method used to demonstrate attenuation of odds ratio as a result of random within-person error is given by Willet [10].
[b] For a comparison of highest quintile *versus* the remaining quintile, quintiles are defined by the exposure distribution of controls. OR, odds ratio.
[c] Within to between-person variance ratio.

coefficients ranged from $r = 0.26$ (copper) to $r = 0.58$ (zinc) ($P \leq 0.003$ for all elements). The within-to-between-person variance ratios ranged from 0.58 for mercury to 3.20 for copper.

The attenuation of odds ratios in a hypothetical case-control study due to the random within-person variability observed for toenail levels of copper and arsenic is shown in Table 3. For a true odds ratio of 3.0 comparing the highest quintile of exposure to the remaining quintiles (quintiles being defined by the exposure distribution of the controls), the observed (attenuated) odds ratio, for the case of toenail arsenic levels as the exposure, was 2.15. For toenail copper levels the observed odds ratio was 1.67. For a true odds ratio of 1.5, the attenuated odds ratios for toenail levels of arsenic and copper were 1.32 and 1.21, respectively.

Fish intake in 1982 was significantly associated with higher toenail mercury levels in 1982–1983 (Spearman's $r = 0.33$; $P \leq 0.0001$); a similar association was observed for 1986 fish intake and toenail mercury levels (Spearman's $r = 0.40$; $P \leq 0.0001$) (Table 4). Correlations between 1986 intake of specific types of fish in relation to toenail mercury levels were as follows: canned tuna fish, Spearman's $r = 0.27$; dark fish, Spearman's $r = 0.19$; shellfish, Spearman's $r = 0.04$; other fish, Spearman's $r = 0.41$.

Discussion

The levels of toenail trace elements we observed in this study are comparable to previous results (12, 15). The significant increases over time in toenail levels of calcium and selenium and decreases in copper, iron, sulfur, and zinc levels suggest that changes in environmental or dietary exposure to these elements may be occurring or that age-related differences in the metabolism of these elements may exist. As both the original and replicate set of nail specimens were analyzed in a paired fashion in the same laboratory run, laboratory drift is not an explanation for these changes over time.

Toenail levels of arsenic and selenium reflect exposure to these elements (5, 6, 16–18); less is known about the reproducibility over time of a single measurement of these elements. Over a 6-year period, we observed reproducibility correlation coefficients of $r = 0.48$ for toenail selenium and $r = 0.54$ for toenail arsenic. These values are only slightly lower than correlation coefficients of 0.6–0.7 typically observed (over a similar time interval) for widely used epidemiological measures such as blood pressure (19). High reproducibility over time of a biomarker known to reflect exposure suggests that the marker has sufficiently low within-person variability that a single measurement will usefully reflect long-term exposure.

Table 4 Relation of fish intake to toenail mercury levels among 127 U.S. women

Frequency of consumption	No. of women	Median toenail mercury levels (μg/g)
Fish intake (1982 questionnaire)		
Almost never	8	0.13[a]
1–3 times/month	25	0.21[a]
Once per week	53	0.30[a]
≥2 times/week	41	0.31[a]
Spearman $r = 0.33$; $P \leq 0.0001$		
Fish intake (1986 questionnaire)		
Almost never	3	0.04[b]
1–3 times/month	12	0.12[b]
Once per week	29	0.20[b]
≥2 times/week	72	0.33[b]
Spearman $r = 0.40$; $P \leq 0.001$		

[a] 1982–1983.
[b] 1988.

In considering toenail trace element levels about which less is known of the sensitivity of these levels to intake, the reproducibility can serve to screen these indicators for their utility as markers of long-term exposure. While a high degree of reproducibility does not prove that a biochemical marker reflects long-term exposure, it identifies markers for which the relation to exposure merits further study. Conversely, a low degree of reproducibility suggests that a biochemical marker does not meaningfully reflect long-term exposure. The relatively high correlations observed for mercury and zinc suggest that toenail levels of these elements may be a good measure of long-term exposure. The lower correlation coefficients seen for elements such as copper, titanium, and chromium suggest that toenail levels of these elements may be poorer indicators of long-term exposure.

We demonstrated the attenuating effects of within-person variability of exposure measurements on estimates of association from a hypothetical case-control study in which a single measurement is assumed to represent long-term exposure. This within-person variability may reflect changes in the exposure to the elements, their metabolism, or incorporation into nails, as well as laboratory error. Attenuation was more severe in the case of toenail levels of copper than in the case of toenail arsenic levels due to the greater within-to-between person variance ratio we observed for copper. However, the within-person variation observed for toenail arsenic levels, which exhibited a relatively high degree of reproducibility, still led to appreciable attenuation of estimates of association. Two approaches to the problem of attenuation of effect estimates due to such variability are: (*a*) to obtain many replicates of the exposure measure for each subject in a study and to use the mean value of each subject as his or her true exposure; and (*b*) to obtain one or more replicates for each member of the study population (or a subgroup of that population) and to use the information about reproducibility of the exposure measurement obtained from such a substudy to correct estimates of association and confidence intervals for the effects of random within-person variability. In situations in which collecting many measurements may be prohibitively expensive or logistically difficult, use of a reproducibility study may be more appropriate, unless there is evidence that the variability is nonrandom (*e.g.*, due to changes in exposure for a segment of the population). A method to correct logistic re-

gression coefficients and their SE for random within-person variability in the exposure measure has been developed for cohort studies (20).

Previous studies have demonstrated a strong dose-response relationship between fish intake and mercury levels in hair (21) and between fish intake and blood mercury levels (22–25). Studies to assess the validity of toenail element levels as indicators of exposure include ecological studies, observational comparisons of exposure *versus* toenail element levels, or intervention studies designed to assess the response to supplementation with an individual element (4). As intervention studies are usually expensive and time-consuming, as well as unethical for elements with toxic effects, observational data are frequently the only source of information on the validity of biomarkers. In this study we took the opportunity to assess the association between fish intake (a major source of dietary mercury) and toenail mercury levels. The positive correlations we observed provide evidence of the suitability of toenail mercury levels to assess mercury intake. The lack of a correlation between intake of shellfish and toenail mercury levels is consistent with observations that mercury levels in shellfish are considerably lower than levels in other kinds of fish (26).

In summary, the relatively high degree of reproducibility over a 6-year period of toenail selenium and arsenic levels (both of which measures are known to reflect dietary intake) suggests that a single measurement of these biomarkers reflects long-term exposure. The high reproducibility of toenail levels of mercury, combined with a positive correlation between mercury and fish intake, suggests that toenail mercury is a reasonably valid and time-integrated measure of mercury exposure. Even when the degree of reproducibility of an exposure measurement is high, substantial attenuation of measures of association in epidemiological studies can result when a single measurement is assumed to represent long-term exposure. Epidemiologists should be aware of the potential for such error and should consider techniques to correct for the resulting attenuation of measures of association.

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Appendix

Appendix[a]

If x is distributed normally[b] with mean μ and variance σ^2, the expected value of x given that x is less than a detection limit value c, is given by:

$$[E(x) \mid x \leq c] = \mu - [(\sigma)\phi(c')/\Phi(c')]$$

where $c' = \frac{(c-\mu)}{\sigma}$, $\phi = \frac{\exp(-1/2\,(c')^2)}{(2\pi)^{1/2}}$, and $\Phi = \int_{-\infty}^{c'} f(x)\,dx$.

[a] The result given here is similar to a result given previously (27).
[b] x represents the $\log_e$-transformed trace element value.

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