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Reliability of low mass toenail samples as biomarkers of chronic metal exposure

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Abstract

BACKGROUND: Toenails are a promising matrix for chronic metal exposure assessment, but there are currently no standard methods for collection and analysis. Questions remain about sample mass requirements and the extent to which metals measured in this matrix are representative of chronic body burden.

OBJECTIVE: This study proposes a method to maximize sample conservation for toenail metals analysis using inductively coupled plasma mass spectrometry (ICP-MS). We demonstrate the reliability of an ~25 mg toenail sample (typically 1–2 clippings) for metals analysis and evaluate the intra-individual variability of multiple metals in this matrix over time in men from the Gulf Long-term Follow-up (GuLF) Study.

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AUTHOR CONTRIBUTIONS

JJYL: conceptualization, methodology, formal analysis, writing—original draft. LJK: formal analysis. MWT: review and editing, data curation. RC: data curation. SGH: data curation. DPS: data management, review and editing. KGL: data management, review and editing. WBJ II: data management. ASD: review and editing. GR: supervision. LSE: supervision, data management. AMR: project administration, supervision.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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METHODS: Toenail samples from 123 GuLF Study participants were collected at two visits 3 years apart and analyzed for 18 elements using ICP-MS. Participants with samples exceeding 200 mg at the first visit ($n = 29$) were selected for triplicate sub-sample analysis. Kendall's coefficient of concordance (W) was used to assess sub-sample reliability and Spearman's correlation coefficients (ρ) were used to evaluate fluctuations in elemental concentrations over time.

RESULTS: Results were not reported for Cd, Co, Mo, Sb, and V (detected in <60% of the samples). There was strong agreement among triplicate samples (Kendall's W: 0.72 (Cu)–0.90 (Cu)) across all elements evaluated, moderate correlations of elemental concentrations (Spearman's ρ: 0.21–0.42) over 3 years for As, Ca, Cr, Fe, Pb, Mn, and Zn, and strong correlations (>0.50) for Se, Cu, and Hg.

IMPACT STATEMENT: This toenail reliability study found that a low-mass (~25 mg) toenail sample (1–2 clippings) is suitable for the determination of most elements using ICP-MS and helps to increase the analytical capacity of limited toenail biospecimens collected in cohort studies. The results highlight differences in the suitability of toenails for chronic metal exposure assessment by element and underscore the need to consider intra-person variability, especially when comparing results across studies. We also provide recommendations for analytical standardization and the partitioning of the total collected toenail sample into multiple analytic sub-samples for future studies using toenail biospecimen for multiple assays.

Keywords

Metals; Biomonitoring; Toenail; Analytical methods

INTRODUCTION

Metals and metalloids, hereafter referred to as "metals" or "elements", are ubiquitous in the environment. While some elements like iron (Fe), Magnesium (Mg), and Calcium (Ca) are essential to human health, others such as lead (Pb), arsenic (As), and mercury (Hg) are toxic at low levels and have been associated with neurobehavioral deficits, immune dysfunction, and adverse cardiovascular outcomes across the life course [1–7]. Humans are exposed to metals through multiple routes with inhalation and ingestion being the most common routes for chronic, low-level exposures. Given that concurrent exposure to a variety of metals through multiple routes of exposure is expected, biomarkers of internal dose are valuable for the determination of metal exposure and related health outcomes.

Toenails have gained favorability as bioindicators of metal exposure in recent years due to their logistic advantages over traditional biospecimens such as blood and urine. They are noninvasive to collect, easy to store, and can accumulate a wide range of metals due to the disulfide bonds in the keratin [8, 9]. Metals that deposit in the toenail matrix are removed from the metabolic processes and can reflect 4–6 months of exposure from up to 6–12 months prior to clipping, dependent on sample length and mass [9–11]. With stable metal concentrations after deposition and no requirements for specialized storage conditions, toenail clippings can be analyzed for elemental content many years after collection [9] and have also been used to characterize metal mixtures exposures, addressing high-priority research areas in metal exposure epidemiology [12, 13].

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Given these advantages, the toenail metal literature has seen rapid growth in recent years. Elemental concentrations in toenail samples have been linked to various health outcomes from high body mass index to breast cancer onset and Amyotrophic Lateral Sclerosis (ALS) [13–16]. Despite the wide utility of this matrix for biomonitoring, there remains significant variability in toenail sampling and analysis protocols, thus limiting the comparability of toenail metal concentrations across study populations. Little is known about the sample mass requirements for toenail metals analysis and even greater uncertainty surrounds whether or which elements measured in the toenail can adequately capture chronic, long-term body burdens in epidemiological studies. A 2019 review by Gutiérrez-González et al. found that studies rarely reported details about sample mass or sampling strategy [17]. One study described a systematic bias in selenium (Se) concentration inversely proportional to sample mass in clippings weighing less than 23 mg [18], but most studies simply reported elemental concentrations by sample mass (μg/g) [17]. Some additionally included sample mass as a variable in regression models [19, 20]. Since the treatment of toenail samples has varied greatly across studies, further research is needed to understand the effect of toenail mass on elemental measurement.

The window of exposure reflected by the elemental concentrations from a toenail sample is determined by toenail growth rate and length of the clipping [21]. With variability in toenail growth rates across participants and the possibility of differential toenail growth rates across toes, toenail samples collected without information about time since last clipping may reflect different windows of exposure across participants [10]. It has been suggested that using toenail clippings from all ten toes would account for an average of the windows of exposure across toes, reflecting integrated exposure over the longest possible exposure period [21, 22]. A smaller subset of studies has also reported reliable toenail metals analysis using only clippings from the big toe, which may allow for a more accurate determination of the time window of exposure while preserving remaining samples for additional assays [23]. However, big toe clippings must be identified and separated at the time of sample collection as it is often unreliable to identify the big toe clippings among the rest after arrival in the analytical laboratory. This introduces another factor for consideration during the sample collection process and limits the practicality of this method for studies that did not separate the big toe clipping at the time of collection.

Moreover, a recurrent challenge with toenail sample collection is inconsistency in the number and type of clippings retained for collection and storage. These challenges are of particular concern in large retrospective cohort studies where samples are self-collected by participants and stored in a study biorepository for many years before sample analysis. While participants in toenail studies are typically instructed to collect clippings from all ten toes, the samples received rarely contain ten uniform clippings. More often, the collected samples vary widely in type with some made up of numerous partial clippings from multiple or all toes and others with just a couple of large clippings representing samples from a subset of toes. Thus, the analysis of the total collected sample without regard to the corresponding exposure window by sample type further complicates comparisons of elemental concentrations across participants with widely varying samples.

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In addition to notable inter-sample variability, questions remain about intra-toenail variability and the potential of using low-mass toenail samples for trace metals analysis to maximize sample conservation for supplemental assays. In addition to their suitability for metals analysis, 20 mg toenail samples have been found to be a convenient means for measuring retrospective cortisol levels [24, 25]. This highlights the feasibility of splitting collected biospecimen for multiple assays and motivates the use of a low-mass toenail sub-sample for metals analysis. Given the wealth of background and confounding information collected in many large cohort studies, prioritizing sample conservation during metals analysis is desirable for maximizing opportunities to answer additional research questions. In particular, the ability to analyze the same collection of toenails for metals and cortisol levels would provide novel opportunities to explore chronic stress as a potential effect measure modifier as well as to examine environmental and psychosocial co-exposures, which are critically under-researched areas in environmental epidemiology [26].

Despite the growing popularity of toenail biospecimens for metals analysis, further research is needed to inform the standardization of toenail sample collection, analysis, and reporting. Moreover, to maximize the value of limited biospecimen samples, it is important to understand whether low-mass toenail samples can be used for reliable metals analysis. This study examines the suitability of a random toenail sample subset (1–2 clippings, or ~25 mg) for metals analysis and evaluates the within person correlation of elemental concentrations in samples across two time points ~3 years apart in a sample of men from the established Gulf Long-term Follow-up (GuLF) Study.

MATERIALS AND METHODS

Study population

In response to the 2010 *Deepwater Horizon* Oil Spill disaster, the National Institute of Environmental Health Sciences (NIEHS) initiated the Gulf Long-term Follow-up (GuLF) Study to evaluate the short- and long-term health effects—including respiratory, neurological, and cardiovascular health endpoints—related to oil spill exposures following the disaster. The GuLF Study follows more than 32,000 participants aged 21 years and older across 5 US Gulf states and throughout the US for potential adverse health effects related to oil spill clean-up exposures [27]. Participants comprise individuals who either worked on the oil spill for at least 1 day or who took mandatory worker safety training but did not work on the spill. Between May 2011 and May 2013, cohort members who lived in Florida, Alabama, Mississippi, Louisiana, and Texas at the time of study enrollment and who spoke English or Spanish were invited to participate in a home visit exam where additional details about oil spill exposures, socioeconomic background, and health history, as well as toenail samples were collected (N = 11,193 home visits completed). Of those who participated in the home visit, 3401 individuals who lived within 60 miles of study clinics in Mobile, Alabama or New Orleans, Louisiana participated in a clinical exam 2 to 4 years later (median 2.8 years), between August 2014 and June 2016. At this visit, trained examiners collected updated health, diet, work history, and residential history data, as well as toenail biospecimens and neurobehavioral test results from 2734 oil spill workers. At both the home visit and clinical exam, participants were instructed to self-collect clippings from all ten

toes. Samples were collected in paper envelopes and stored in the GuLF Study biorepository until analysis in 2021. Details about study enrollment and cohort follow-up are available elsewhere [27, 28].

Inclusion criteria: to maximize analytical overlap with existing GuLF Study biomarker studies, 679 participants with previously measured liver and kidney function/injury biomarkers (selected based on oil spill exposure) were selected for toenail metals analysis. To mitigate metal exposure misclassification, we further excluded self-reported current smokers. This resulted in a final analytical population of 428 participants eligible for toenail metals analysis. Given previously reported biases in metal concentration in toenail samples weighing less than 23 mg [18], and wide variability in toenail clipping amounts collected in the study, we set a target mass of 25 mg to achieve greater consistency in analytical sample mass. Seventeen samples weighing as little as 20 mg were included to maximize the analytical sample size but samples that had a total mass <20 mg prior to cleaning were excluded because of limit of detection concerns with the analytical instrument based on method development test runs (not shown). In total, 413 participants contributed roughly 25 mg (1–2 clippings) of their clinical exam toenail samples for metals analysis. Of these 413 participants, we selected 123 geographically stable participants (i.e., remained at the same residential address between visits) for additional home visit toenail metals analysis to answer questions about potential fluctuations inter-toenail elemental content over time. This resulted in 123 participants with paired toenail metal concentrations from both toenail collection time points (home visit and clinical exam) roughly 3 years apart. Of the 123 participants providing toenail samples at the home visit, 29 participants with samples >200 mg were selected for triplicate 3 × 25 mg sub-sample analysis to test intra-toenail variability (Fig. 1).

Metals analysis

Toenail samples were analyzed for 18 metal/metalloids (aluminum (Al), antimony (Sb), arsenic (As), cadmium (Cd), calcium (Ca), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), magnesium (Mg), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), vanadium (V), and zinc (Zn)) using inductively coupled plasma mass spectrometry (ICP-MS). Roughly 25 mg, or 1–2 clippings, from the total sample were randomly selected for metals analysis. Individual clippings were not further divided for this analysis to achieve the target 25 mg mass. Thus, some samples undergoing analysis were greater than 25 mg, corresponding to the weight of the smallest single toenail clipping 25 mg in the collected sample.

Selected analytical sub-samples underwent a cleaning process modified from Tehrani et al., involving a nonpolar solvent, a polar solvent, and a detergent [29] which should not leach endogenous concentrations of elements that irreversibly bind in the toenail matrix [30]. Samples were sonicated for 15 min in 10 ml 30% acetone, rinsed with Milli-Q water, and sonicated again for 15 min in 10 ml 1% Triton X-100 solution before being rinsed five times with Milli-Q water and dried at room temperature for 48 h or until stable mass was observed under a positive pressure clean air hood (Envirco Corp, Albuquerque, NM). All reagents

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used were purchased at the highest available purity. After the samples were cleaned and dried, they were reweighed to determine the final analytical sample mass.

Cleaned toenail samples were digested using a method adapted by the Dartmouth Trace Element Analysis Core for low-mass biological samples [14]. Samples were pre-digested with 0.5 ml nitric acid (HNO_3) optima overnight prior to open vessel microwave-assisted digestion. Samples were then loosely capped and heated with 10 min ramp to temperature and 15 min hold time at 110 °C in a MARS 6 microwave (CEM Corporation, Mathews, NC). After cooling, 0.2 ml of hydrogen peroxide (H_2O_2) optima was added to each sample and the microwave cycle was repeated. After the second microwave cycle, samples were cooled and diluted 1:9 with 0.5% hydrochloric acid (HCl) for analysis by ICP-MS (Agilent 8800 ICP-MS Triple Quad; Agilent technologies, Inc., Santa Clara, CA). ICP-MS gas modes for each element are provided in Supplementary Table 1.

Toenail samples were analyzed in seven analytical batches, with triplicate samples analyzed in the same batch. Data quality was monitored via multipoint calibration curves for each analyte at the beginning and end of each batch, analysis of laboratory and digestion blanks, duplicates, spikes, and comparison with two reference materials: human hair Japan NIES #13 (National Institute for Environmental Studies, Ibaraki, Japan) and caprine horn NYS RM 1801 (New York State Department of Health Wadsworth Center, Albany, NY) [29]. The average between-batch coefficient of variation across elements was 11% and ranged from 3% (Pb) to 27% (Sb). The limit of detection (LOD) for each element was calculated using three times the standard deviation of digestion blanks ($N = 7$) for each batch. The average LOD for each element across batches ranged from 0.00002 μg/g for Cd to 0.3 μg/g for Ca (Table 1). Samples below LOD were imputed with the batch-specific LOD divided by 2 [31].

Statistical methods

Toenail metal concentrations were $\log_{10}$ transformed to address skewness in the concentration distributions (Supplementary Fig. 1). Alternative methods to transform the data (square, square root, cube, cube root, standard deviation standardization) were also attempted but resulted in inferior normal distributions compared to $\log_{10}$ transformation. While the concentration distributions after log transformation were closest to normal, none of the elements except for Ca and Se passed the Shapiro-Wilk test of normality. Thus, non-parametric methods were the primary means used to determine sub-sample reliability and intra-individual variability over time.

Sub-sample reliability.—To assess the suitability of a 25 mg toenail sub-sample for metals analysis, triplicate sub-samples were selected from 29 large samples (>200 mg) from the home visit for the determination of sub-sample agreement using Kendall's coefficient of concordance (Kendall's W).

Kendall's W is a non-parametric, rank-based statistic that can be used to assess the degree to which a group of variables provides a common ranking for a set of objects [32, 33]. The W statistic produces an estimate of the variance of the row sums of ranks, divided by the maximum possible variance, which results in a statistic ranging from 0 to 1 [33]. For this

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data, a W of 1 indicates that among each of the triplicate measurements, the ordering of concentrations among respondents is the same, while a W of 0 indicates there is no trend of agreement between measurements, and the measurements are random. Kendall's W is calculated using Eq. (1):

$$W = \frac{12S}{p^2(n^2 - n)} \tag{1}$$

where S is the sum of squares over the row sum of ranks, n is the number of participants, and p is the number of replicate measurements

Despite non-normality in the data after transformation, linear mixed effect models were fit using the log-transformed data for comparison of the above-mentioned Kendall's W with the more widely used and easily interpretable intraclass correlation coefficient (ICC), which is calculated using values for intra- and inter-person variability from a linear mixed effects regression output [34]. Analytical batch and sample mass effects were also evaluated as predictors of metal concentration in the linear mixed model. The linear mixed model is described using Eq. (2):

$$\log 10(\text{Metal})_{ij} = \beta 0 + \beta 1(\text{Mass})_{ij} + \beta 2(\text{Batch})_{ij} + b3(\text{ID})_i + \varepsilon_{ij} \tag{2}$$

where Mass is the mass of the sample, Batch is the analytical batch [1–7], and ID is the participant ID.

Sub-sample variability and elemental concentration.—A simulation was performed to assess the effect of sub-sample variability on overall elemental concentration measured by the average of the triplicate samples. Using the sample distributions from the 29 participants with replicate measurements, we estimated the association of a random single toe clipping sample (from distributions with varying Kendall's W) with the average concentration of the triplicate samples. This is the comparison between concentrations measured using a 25 mg toenail sub-sample and the larger sample. Since concentrations measured in the toenail are often orders of magnitude different by element, the log-transformed data were first scaled and standardized to have mean 0 and variance 1 among each sample (1, 2, and 3) to facilitate comparisons across elements with different concentration ranges. Then, for each element, the following process was repeated for 10,000 iterations:

1. For each subject $i = 1, \ldots, 29$ determine $\bar{x}_i$: the mean concentration.
2. For each subject $i = 1, \ldots, 29$ set $y_i = \bar{x}_i$.
3. For each subject $i = 1, \ldots, 29$ randomly sample one of the three measurements, denote this value $x_{i,\text{ rand}}$.
4. Fit a linear model $y_i = \beta x_{i,\text{rand}} + \epsilon_i$; calculate $\hat{\beta}$; store.

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The estimated effect size $\bar{\hat{\beta}}$ and variance of the effect size $\mathrm{var}(\hat{\beta})$ were calculated as the mean and variance, respectively, of the 10,000 stored $\hat{\beta}$.

Intra-individual variability over time.—The intra-individual variability of toenail metal content over time can be evaluated by estimating within-person correlation among samples collected across different time points. This concept has been referred to as "reproducibility over time" or "stability over time" in previous toenail biomarker research [17, 35, 36]. The degree of correlation informs the extent to which metal exposures in this matrix can reflect chronic, long-term body burden in this population. Spearman's rank correlation coefficients were calculated using concentrations from the 123 paired samples collected from participants at home visit and clinical exam 2–4 years apart (median: 2.8 years) (Fig. 1). Significance values (p) for Spearman's correlation coefficients were obtained through permutation tests. Median concentration changes between visits were calculated for each element to summarize fluctuations over time.

RESULTS

Paired toenail samples from two time points for 123 participants were analyzed for a panel of 18 metals/metalloids for this reliability study. Of the 123, a subset of 29 participants with large toenail samples (>200 mg) from the home visit also provided triplicate (3 × 25 mg) samples for sub-sample reliability analysis. As expected, based on the locations of the clinical exam sites, most participants were from Alabama ($n = 60, 49\%$) and Louisiana ($n = 39, 32\%$) with the rest from Mississippi ($n = 14, 11\%$) and Florida ($n = 10, 8\%$). Forty-four percent ($n = 54$) of participants identified as Black or African American, 50% ($n = 61$) as White, 3% ($n = 4$) as multi-racial, and 3% ($n = 4$) as other. Median age at the home visit was 46 years (range: 21–65) and median age at the clinical exam was 49 (range: 23–69) (Supplementary Table 2).

The total masses of toenail samples collected in the GuLF study varied widely (range: 9–590 mg prior to cleaning) despite standardized collection instructions. Some samples contained only 1 or 2 clippings and others contained upwards of 20 partial clippings. On average, 3.4 mg of mass was lost in the cleaning process. While sub-samples greater than 20 mg were selected for analysis the final washed toenail samples undergoing metals analysis ranged from 12.5–83.1 mg (median: 25.3 mg).

Al, Ca, Co, Fe, Pb, Mg, Mn, Hg, and Ni were detected in 100% of the samples. As, Se, and Zn were detected in 99% of the samples. Sb, Cd, Co, Mo, and V were detected in less than 60% of the samples, at 58%, 23%, 35%, 17%, and 21%, respectively. Given their variable detection frequencies in our samples and analytical noise near the LOD for detectable samples, the latter group of elements was excluded from subsequent analyses of sub-sample agreement and intra-individual variability over time. Summaries of the LODs and elemental concentrations are provided in Table 1. Sample mass and analytical batch were not significant predictors in the linear mixed models and did not appear to have significant effects on measured toenail metal concentrations (not shown).

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Sub-sample reliability

Sub-sample agreement was strong (Kendall's $W > 0.70$) for all elements that were detected in more than 60% of all samples. Kendall's W for the 29 participants with triplicate samples ranged from 0.72 (Cr) to 0.90 (Cu). Linear mixed effects models fit to assess intra- and inter-person variability and corresponding ICCs resulted in similar rankings of sub-sample agreement by element. A comparison of sub-sample reliability between both methods is presented in Table 2. The strong inter-sub-sample agreement observed in this study indicates that the variability in elemental concentration across participants is far greater than within sample variability by toenail.

Cu had both the highest Kendall's W (0.90) and ICC (0.88) while Cr had the lowest Kendall's W (0.72) and second lowest ICC (0.64). Scatterplots comparing sub-samples 1 to 2, 1 to 3, and 2 to 3 for both metals show strong visual agreement of measured Cu concentrations (green) between triplicate samples along the 1:1 line in gray and wider distribution of Cr concentrations (orange) around the line of agreement (Fig. 2). Compared to Cu, for which all samples were above the LOD, 7 samples were below LOD for Cr (indicated with triangles) and overall Cr concentrations were consistently closer to the LOD, for which more measurement noise is expected. Scatterplots of sub-sample agreement for all other elements analyzed are provided in Supplementary Fig. 2.

Sub-sample variability and elemental concentration

The attenuation of the association between a random toenail sub-sample and the mean of the triplicate samples due to inter-sub-sample (or intra-toenail) variability for each element is shown in Table 3. Assuming no inter-sub-sample variability or measurement error, the true association between a random toenail sub-sample and the average of the 3 replicate samples would be exactly equal to 1. But, for elements with varying sub-sample reliabilities (Kendall's W: 0.72–0.90), the associations range from 0.71–0.91. However, with only three samples, the level of attenuation does not exactly trend the reliability of the sub-sample. For example, while Cu has the highest Kendall's W and ICC at 0.90 and 0.86, respectively, the association of a random sub-sample with the average of the three sub-samples is 0.86. Meanwhile, Pb, which has lower sub-sample reliability scores (Kendall's $W = 0.88$, ICC $= 0.80$), exhibits a less attenuated association between a random sub-sample and the average of triplicate samples at 0.91. Despite the variability in the ordering of attenuated associations by element, even the elements with the lowest sub-sample reliability scores (Cr, Zn, and Ni) exhibit associations of 0.81, 0.81, and 0.71, respectively. Thus, the elemental concentration differences between the sub-sample and the average of a larger combined sample do not exceed 29% and for most elements is less than 19%.

Intra-individual variability over time

The median time between toenail collections was roughly 3 years (34 months, range: 20–48 months). Overall, toenail metal concentrations decreased from the first (home visit) to the second (clinical exam) timepoint. Changes in elemental concentrations over time, presented as the median difference between visits, as well as Spearman's correlation for toenail

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concentrations between visits by element, are shown in Table 4. Toenail Hg concentrations were the most stable over time, with a median change of −0.049 μg/g over the 2.8 years and good Spearman's correlation of 0.59 between visits. Similarly, toenail Cu and Se showed relative stability over time, with Spearman's correlations of 0.55 and 0.52, respectively. Cr, As, Ca, Fe, Mg, Pb, Zn, and Mn all had moderate correlations over time ranging 0.21–0.42. Ni and Al exhibited the lowest Spearman's correlations, at 0.14 and 0.19, respectively (Table 2). Density plots comparing the distribution of toenail elemental concentrations between the two visits are provided in Supplementary Figs. 3.1 and 3.2.

DISCUSSION

Given limited biospecimen availability in most cohort studies, the use of low-mass toenail samples maximizes sample conservation for future study substantially increases leverage to negotiate sample release, and allows for the inclusion of more subjects with low-mass samples. In many cases, these benefits are likely to substantially outweigh modest increases in the exposure measurement variability. In this study of toenail sub-sample reliability for metal analysis, we found strong agreement among random ~25 mg triplicates in 29 participants across elements, moderate correlation over roughly 3 years among 123 participants for Cr, Fe, As, Pb, Zn, Mg, Ca, and Mn, and strong correlation for Cu, Hg, and Se.

One to two toenail clippings or roughly 25 mg was sufficient for detection using ICP-MS in above 95% of our samples for all elements tested except for Cd, Co, Mo, Sb, and V. All of these low-detect (detected in <60% of samples) elements exist in trace levels in the environment, and all but Cd have been reported with low detection frequency in prior studies [8, 17, 37, 38]. The exclusion of smokers in the analytical cohort may contribute to the largely undetectable toenail Cd concentrations in this study; however, we cannot preclude limited sample mass as a potential reason for low Cd detection. While sample mass was not a significant predictor of toenail elemental concentration for any of the elements with low detection, given the purposefully small sample mass and limited sample size, it is possible that a sample mass threshold above the amount used in this study is needed for the analysis of Cd.

Samples collected by the Gulf Study varied widely in mass (9–590 mg) despite standardized instruction for participants to collect clippings from all ten toes. With such substantial variability in sample collection, there can be significant variability in the exposure windows corresponding to sample type (i.e., more specific exposure windows for big toe clippings and a more variable mix of exposure windows for samples containing clippings from remaining toes). However, in cases where the exposures of interest are relatively consistent over time, elemental concentrations across different exposure windows are likely to be comparable [39]. Thus, elemental concentrations obtained from a subset of 1–2 clippings should not be substantially different from concentrations reported from an average of all collected clippings and could be used to conserve biospecimen for future study.

All elements detected in more than 60% of toenail samples in this study demonstrated good sub-sample agreement (Kendall's $W > 0.70$) but greater variability was observed for

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elements with concentrations closer to the LOD such as Cr (Fig. 2). This is consistent with knowledge of greater analytical noise near the LOD [40] and suggests that the analysis of trace elements that typically exist at low concentrations in the environment may benefit from the analysis of a larger sample. However, other studies have reported higher proportions of non-detects for these elements even when using the full set of toenail samples [17]. Thus, increasing the analytical sample mass to achieve slight improvements in sub-sample reliability for Cr may not justify changes to the sampling protocol. Results from the simulation showing the effect of sub-sample variability on overall elemental concentration bias show that sub-sample variability could result in differences from 9% for Pb (Kendall's $W = 0.88$) to 29% for Ni (Kendall's $W = 0.75$) when compared to the average of three replicate measures. While analytical reasons for the inter-element variability are beyond the scope of this work, previous studies have reported variability in the determination of Ni in keratinized material due to the existence of spectral interferences for this element [41]. Sub-sample variability may also be explained by potential contamination which remains a limitation of using toenail clippings for metal biomonitoring. While the contamination concern is not specific to the low-mass sampling method presented here, the lack of validated sample cleaning protocols for the toenail matrix means that it is often difficult to determine endogenous elemental concentrations from exogenous contamination. While outlier values observed in our samples (Supplementary Fig. 1) could be influenced by potential contamination, we chose not to remove outliers from the analysis as their inclusion provides the most conservative estimates of sub-sample reliability. Our results show that despite potential exogenous contamination, there is strong agreement among triplicate samples indicating that reported concentrations are driven primarily by endogenous concentrations rather than exogenous contamination.

Compared to other established metal biomarkers often used in epidemiological research, a low-mass toenail sub-sample is at least comparable for the measurement of multiple metals in a single matrix in terms of sample variability over time. Spot measurements of As, Cu, Pb, and Ni in urine are reported to have ICCs between 0.10 (Pb) and 0.41 (As) for samples collected days apart [42]. The same study by Wang et al. also reported the number of repeated spot samples needed to estimate participant-specific mean within 20% of the true value to range from four samples for As up to 20 samples for Pb. In comparison, the variability introduced by using a single low-mass toenail sub-sample in our study was within 20% of the mean value for all elements tested except for Ni (29%). Other studies of Pb concentration in overnight urine samples undergoing creatinine and specific gravity corrections have reported ICCs of 0.66 and 0.88, respectively [43]. These findings are comparable to the values we report for Pb using a low-mass toenail sub-sample $ICC = (0.80)$ and only slightly lower than values reported using blood Pb measurements, which typically have ICCs around 0.9 or higher [43–45].

It is important to note that only three sub-samples were used to assess sub-sample reliability and the value of the larger, comparison sample was based on an average of measurements of the three sub-samples rather than on a single measurement of the larger sample. It is expected that the analysis of independent sub-samples would be subject to greater analytical variability than if the entire sample was analyzed together. If sub-samples were pooled

for each participant, we would expect a more stable average and smaller biases in the sub-sample concentrations. Thus, our replicate sampling strategy allowed us to provide conservative estimates of sub-sample reliability.

Increased measurement variability introduced by the smaller toenail sub-sample may cause some attenuation of effect estimates of exposure-outcome relationships. However, for many environmental metal exposure studies, the rank ordering of participant exposure levels is of primary interest. In these cases, and especially when subjects are placed into quantiles for analysis, the use of toenail sub-samples, which largely preserve rank ordering, is acceptable. However, the low mass sub-sample analysis method presented here may not be suitable for all applications of toenail metals analysis. More specific determinations of the exposure windows are typically required for dietary exposure validation and occupational studies of specific exposure tasks. In these cases, it may be beneficial to focus on collecting and separating the big toe clipping or ask participants to collect samples at a standardized time since last clipping to achieve greater consistency of exposure windows in the collected toenail samples. Thus, the low-mass analysis method presented here may be limited in utility to environmental epidemiological studies or occupational studies in which the metal exposures of interest can reasonably be assumed to be high and/or continuous.

Toenails are often assumed to be good measures of chronic body burden since elemental concentrations in toenail clippings are averaged over 4–6 months [10]; however, not all elements measured in the toenail have the same value as markers of chronic body burden. The extent of the chronic body burden assumption beyond this range varies by element and is dependent on the nature of the exposure as well as the storage, distribution, and excretion of the element in the body. If exposures were exactly equal over time, we might expect highly reproducible concentrations over many years but, in reality, there are often fluctuations in exposure patterns even for continuous exposures. We demonstrate here that certain elements have greater correlation over roughly 3 years than others which affects the utility of toenail metal concentrations and their interpretation in epidemiologic studies.

All elements detected in more than 60% of the samples, except for Ni, were significantly positively correlated over 3 years. Cu, Hg, and Se had high Spearman's correlations ($\rho > 0.5$) indicating that these elements had relatively comparable concentrations over time and may be justifiable measures of chronic body burden in the toenail over this period. Depending on the outcome of interest, this could have important implications for the interpretation of cross-sectional studies in which ambiguous temporality of the exposure measurement is a major hindrance to causal inference. Low variability or relative stability of elemental concentrations over multiple years could suggest consistency in a dominant single source of exposure. For Hg (Spearman's $\rho = 0.59$), and Se (Spearman's $\rho = 0.52$), exposure sources are likely to be through the diet which can be relatively stable in adults due to social or cultural factors [46]. Total Hg captured in the toenail is mostly comprised of methylmercury which, in this coastal population, is likely related to frequent seafood consumption. Se concentration in the toenail is known to correlate strongly with Se supplementation, thus, stability in toenail Se concentration may be driven by the use of nutritional supplements [22].

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On the other hand, Cr, Fe, As, Pb, Zn, Mg, Ca, and Mn exhibited moderate correlations over time (0.21–0.42), suggesting that exposures to these elements may not be consistent over time. This may be explained by the fact that adults are typically exposed to Cr (Spearman's $\rho = 0.21$), As (Spearman's $\rho = 0.29$), and Pb (Spearman's $\rho = 0.33$), through industrial or anthropogenic sources, many of which may not follow consistent exposure patterns over many years due to changes in industrial operations or regulations. The remaining elements in this category Fe (Spearman's $\rho = 0.25$), Zn (Spearman's $\rho = 0.33$), Mg (Spearman's $\rho = 0.38$), Ca (Spearman's $\rho = 0.40$), and Mn (Spearman's $\rho = 0.42$), are essential metals for which relatively stable exposure from the diet may be modified by environmental exposures to these elements. Lastly, Ni and Al exhibited low correlation (0.14 and 0.19, respectively) over time, suggesting that exposure to these metals may be episodic or that samples may be more susceptible to exogenous contamination of these elements. A previous report showing greater spectral interference for Ni in particular [29] may also suggest that toenail is simply a suboptimal matrix for assessing Ni exposure.

Overall decreasing toenail metal concentrations over time is consistent with some previous reports of inverse relationships between toenail metal levels and increasing age [36, 47]. However, the generalizability of our findings is limited since elemental variability over time is highly dependent on the magnitude and pattern of the external exposures in this specific population, which in this study is limited to men.

In cases where the big toe clipping was not identified and separated at the time of sample collection, using a random subset of one's total toenail sample to achieve standardized analytical mass may be a suitable option to maximize sample conservation while still comparing reasonable exposure windows within the analytical population. Wide adoption of low mass sample standardization for analysis may also improve inter-study comparability by providing a method that is feasible to implement regardless of how samples were collected and stored. Given the variability in toenail sample types collected in cohort studies, studies using toenail samples for metals analysis should prioritize sample description and reporting and provide details about sample mass ranges, and averages. It is also critical for studies to consider the various use cases of toenails for metal analysis as well as the impact of low-mass samples on the analysis and corresponding interpretation of study findings. Future studies collecting toenails for metals analysis may also consider standardizing time since last clipping during study design in efforts to standardize exposure windows for comparison across participants. At the very least, it may be useful to ask participants about time since the last clipping or the general frequency of toenail clipping behaviors to estimate relevant exposure windows for the collected samples.

CONCLUSION

This study demonstrates the suitability of an ~25 mg toenail sample subset (1–2 clippings) for metals analysis and shows good intra-individual stability of toenail Hg, Cu, and Se and moderate intra-individual stability of As, Ca, Cr, Fe, Pb, Mn, and Zn concentrations over 3 years among participants from the NIEHS GuLF Study. We show the advantages of this method to maximize sample conservation and increase the analytical capacity of limited biospecimen for environmental epidemiology studies where the exposure is hypothesized to

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be chronic and relatively continuous. Cd detection is poor in the low-mass sub-sample of non-smokers and Al and Ni concentrations do not appear to be correlated across timepoints 3 years apart. Nonetheless, our findings highlight considerations of elemental variability across toenail sample types that are critical for inter-study comparison and provide context to help future studies decide how to partition their samples for multiple assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY

The data and code can be requested by email to the corresponding author.

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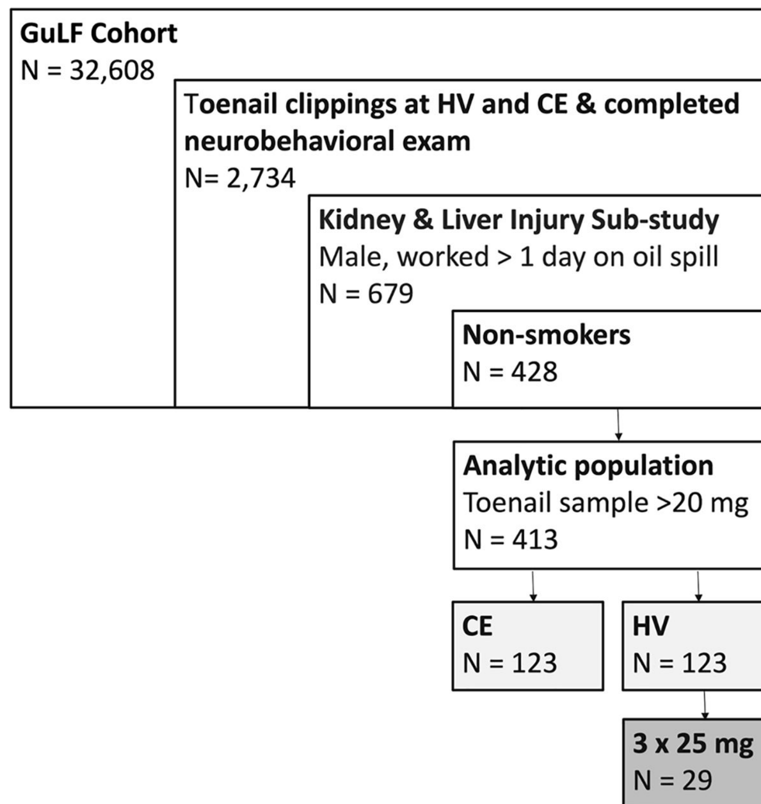

Fig. 1. Flowchart of participant selection for toenail metal analysis.

HV indicates home visit and CE indicates clinical exam. Data from the light gray boxes were used to address reproducibility of toenail metal concentrations across home visit and clinical exam time points 2–4 years apart. Data from the dark gray box was used to determine the reliability of the 25 mg sub-sample.

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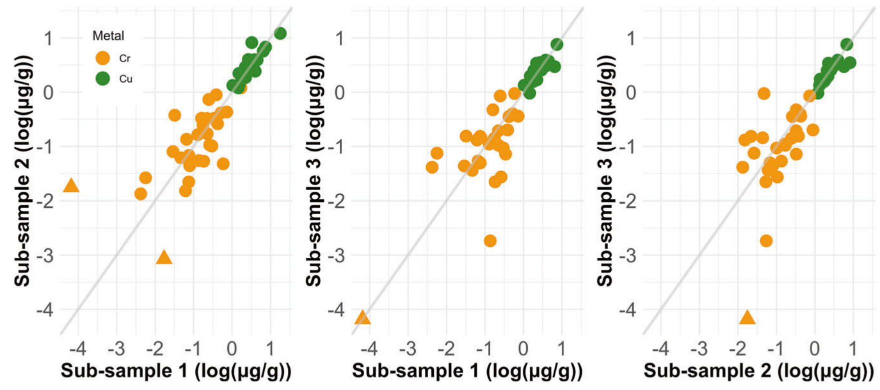

Fig. 2. Correlation between sub-sample metal concentrations (log10 (μg/g)) for Cu ($W = 0.90 \mid \mathrm{ICC} = 0.88$) and Cr ($W = 0.72 \mid \mathrm{ICC} = 0.64$).

The gray line indicates the line of agreement (1:1) between sub-samples. Triangular points indicate samples for which at least one was below LOD.

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Table 1.

Summary of LODs and elemental concentrations (μg/g) from HV toenail samples (n = 123).

Metal	LOD[a] (μg/g)	Percent detection	Geometric mean (GSD) (μg/g)	Min (μg/g)	Max (μg/g)
Aluminum	0.1	100	12 (2.5)	0.74	63
Antimony[b]	0.0002	58	0.0029 (35)	<LOD	0.54
Arsenic	0.0003	99	0.059 (3.5)	<LOD	5.2
Cadmium[b]	0.00002	23	0.00011 (20)	<LOD	0.39
Calcium	0.3	100	1110 (1.9)	16	4125
Chromium	0.002	95	0.20 (7.7)	<LOD	42
Cobalt[b]	0.00002	35	0.00015 (18)	<LOD	0.061
Copper	0.003	100	3.5 (1.6)	0.14	15
Iron	0.03	100	14 (2.3)	2.7	112
Lead	0.0003	100	0.23 (4.1)	0.003	11
Magnesium	0.02	100	127 (1.7)	5.9	649
Manganese	0.0003	100	0.43 (3.1)	0.03	4.8
Mercury	0.0006	100	0.18 (3.4)	0.0002	3.3
Molybdenum[b]	0.0002	17	0.00068 (6.8)	<LOD	1.2
Nickel	0.0006	100	0.61 (5.2)	0.0003	31
Selenium	0.0005	99	0.69 (2.1)	<LOD	4.6
Vanadium[b]	0.001	21	0.000071 (23)	<LOD	0.12
Zinc	0.007	99	98 (2.6)	<LOD	858

[a] LOD varied by batch—average LOD across batches is shown.

[b] Detected in less than 60% of samples.

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Table 2.

Comparison of Kendall's concordance coefficient (*W*) to intraclass correlation coefficient (ICC).

Metal	Kendall's concordance coefficient (*W*)	ICC_{log10}
Aluminum	0.77	0.78
Arsenic	0.79	0.73
Calcium	0.80	0.71
Chromium	0.72	0.63
Copper	0.90	0.87
Iron	0.78	0.72
Lead	0.88	0.79
Magnesium	0.81	0.74
Manganese	0.85	0.64
Mercury	0.85	0.70
Nickel	0.75	0.56
Selenium	0.86	0.79
Zinc	0.87	0.77

ICC_{log10} represents the ratio of between sub-sample variability to total variability using log(10) transformed toenail metal concentrations.

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Table 3.

Attenuation of the association between sub-sample concentration and the mean concentration of three repeated sub-samples due to sub-sample variability.

Metal	Kendall's *W*	ICC	True association, 1.0	Standard error (SE)
Aluminum	0.77	0.76	0.81	0.08
Arsenic	0.79	0.76	0.80	0.12
Calcium	0.80	0.71	0.84	0.09
Chromium	0.72	0.64	0.81	0.13
Copper	0.90	0.86	0.86	0.09
Iron	0.79	0.73	0.81	0.09
Lead	0.88	0.80	0.91	0.10
Magnesium	0.81	0.75	0.87	0.06
Manganese	0.85	0.72	0.84	0.15
Mercury	0.85	0.71	0.80	0.15
Nickel	0.75	0.57	0.71	0.13
Selenium	0.85	0.79	0.89	0.07
Zinc	0.72	0.64	0.81	0.13

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Table 4.

Median and median changes in toenail metal concentration over time and Spearman's correlation between visits (n =123).

Metal	Median concentration HV (μg/g)	Median concentration CE (μg/g)	Median difference between visits (μg/g)	Spearman's correlation	*p* value
Aluminum	13	8.6	−2.4	0.19	0.04
Arsenic	0.062	0.041	−0.018	0.29	0.002
Calcium	1180	1013	−129	0.40	0.0002
Chromium	0.23	0.18	−0.047	0.21	0.02
Copper	3.4	3.4	−0.061	0.55	0.0002
Iron	12	12	−0.80	0.25	0.005
Lead	0.22	0.17	−0.038	0.33	0.0004
Magnesium	122	103	−9.3	0.38	0.0002
Manganese	0.43	0.39	−0.019	0.42	0.0002
Mercury	0.20	0.15	−0.049	0.59	0.0002
Nickel	0.46	0.21	−0.15	0.14	0.1
Selenium	0.73	0.75	0.0080	0.52	0.0002
Zinc	103	98	−1.5	0.33	0.0008