Toenail Arsenic Concentrations, *GSTT1* Gene Polymorphisms, and Arsenic Exposure from Drinking Water

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Abstract

Toenail arsenic (As) concentrations were evaluated as a biomarker of inorganic As (As_{in}) exposure in a population residing in an As-endemic region of Bangladesh. Drinking water and toenail samples were collected from 48 families (n = 223) every 3 months for 2 years and analyzed for As using inductively coupled plasma-mass spectrometry. Drinking water collected 3, 6, and 9 months before each toenail sample collection was combined into a weighted lagged exposure variable. The contribution of each water sample to the measured toenail As concentration was estimated using maximum likelihood that accounted for fluctuations in drinking water exposure and toenail growth. The best model attributed 69%, 14%, and 17% of the toenail As content to drinking water exposures that occurred 3, 6, and 9 months before toenail collection [95% confidence intervals (95% CI), 0.46-0.97, 0.00-0.31, and 0.03-0.35, respectively]. Generalized additive mixed models using penalized regression splines were employed to model the data. Below a drinking water concentration of 2 μg As/L, no relationship between drinking water As and toenail As concentrations was observed. Above this concentration, toenail As content increased in a dose-dependent fashion as drinking water As increased. Age was a significant effect modifier of drinking water As exposure on toenail As (β = 0.01; 95% CI, 0.002-0.02). Individuals possessing *GSTT1*-null genotypes had significantly more As in their toenails in contrast to *GSTT1* wild-type individuals (β = 0.11; 95% CI, 0.06-0.2). Therefore, it seems that *GSTT1* modifies the relationship between As_{in} exposure and toenail As_{in} content. (Cancer Epidemiol Biomarkers Prev 2005;14(10):2419–26)

Introduction

Ingestion of inorganic arsenic (As) is a significant public health hazard in Bangladesh where an estimated 25 million to 57 million people are chronically exposed to inorganic As in their drinking water. This is a consequence of public health initiatives begun in the 1970s that were designed to decrease the morbidity and mortality associated with waterborne disease by introducing pathogen-free groundwater through the installation of shallow tube wells (1). In the early 1990s, physicians began to document cases of As-associated skin lesions in West Bengal and Bangladesh. Groundwater was confirmed as the source of As after a national survey of 10,991 tube wells found that 59% of the tube wells sampled exceeded the Bangladesh drinking water standard of 50 μg As/L and that 73% exceeded the WHO recommended drinking water guideline of 10 μg As/L (2). It is estimated that 95% of the drinking water in Bangladesh is currently provided by tube wells (3).

Although consumption of As-contaminated drinking water is the primary route of As exposure for most individuals, additional exposures can occur through the mining and smelting of metal ores, manufacturing and application of pesticides and wood preservatives, using medicines and homeopathic remedies, and ingesting As-contaminated food products (4). Chronic exposure to As is associated with many negative health effects, including the development of noncancerous skin lesions (5), neuropathy (6, 7), peripheral vascular diseases (8), skin (9, 10), bladder (11), and lung cancers (12).

Forty-five percent to 85% of ingested As are excreted in urine within 1 to 3 days of exposure, making urinary As concentrations a useful biomarker of recent exposure but a poor measure of historical exposures (13-15). However, As has a high affinity for sulfhydryl groups resulting in its accumulation in keratin-rich tissues, such as hair, skin, fingernails, and toenails (16-18). Once bound to keratin, As is isolated from metabolic activity. Thus, any As detected in the keratin matrix will reflect only those conditions that occurred during its formation (19). Of these keratin-based biomarkers, toenails are better protected from external environmental contaminants and chemical treatments, making them an ideal biomarker candidate.

Depending on the length of the nail and its growth rate, it can take several months and up to 1 year for the keratin matrix to grow to the free edge of the nail plate (20). Therefore, toenail clippings act as a time-integrated measure of As exposure. Biomarkers of exposure are useful in epidemiologic studies to verify subject-specific exposures and as an index of internal dose (21, 22). To use toenail As in these capacities, it is important to evaluate the relationship between toenail As and environmental exposure as well as factors that could influence this dose-effect relationship.

Consequently, a longitudinal study in an As-endemic region in Bangladesh was conducted to examine the association between As-contaminated drinking water and biomarker response. This article presents the dose-effect relationship between toenail As content and As-contaminated drinking water. Selected physiologic and sociodemographic characteristics were evaluated to determine their influence on biomarker response, including age, gender, smoking status, and betel nut chewing behaviors. Genetic polymorphisms in glutathione *S*-transferase genes are also evaluated because glutathione is a necessary enzyme in As metabolism (23).

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Materials and Methods

Site and Participant Selection. Participants were recruited through a series of community meetings held in three villages (village 5, village 6, and village 12) in Pabna, Bangladesh, which form part of the catchment area of the Pabna Community Clinic. Although As-affected patients are reported in this district, Pabna is believed to be only moderately As affected. A previous survey found that the mean groundwater As concentration in this district was 32 μg/L (24). Current research indicates that the elevated As concentrations in the groundwater is driven by the microbial dissolution of As-bearing mineral phases, primarily iron oxyhydroxides (25, 26).

Overall, 248 individuals comprising 50 families were recruited to participate in a repeated-measures study to characterize biomarker response. Subjects were eligible for this study if they were long-term residents of Pabna, obtained their drinking water from tube wells, and received primary health care from the Pabna Community Clinic, an affiliate of Dhaka Community Hospital. During the initial visit in September 2001, a behavioral and demographic questionnaire was administered and a blood sample was collected. Researchers visited participants at their homes every 3 months to collect urine, toenail, and water samples. The data used in this analysis were collected from April 2002 to September 2004.

The institutional review boards at Harvard School of Public Health and Dhaka Community Hospital approved the protocol for this study. Informed consent was obtained from all adult participants before participation and parental consent was obtained for all participants ages <18 years.

Toenail Collection and Analysis. Toenail clippings were collected from all toes and pooled to ensure sufficient mass for analysis. Toenail clippings were prepared as described by Chen et al. (27). Briefly, external contamination was removed from nails by sonicating samples in a 1% Triton X-100 solution (Sigma-Aldrich, Inc., St. Louis, MO) for 20 minutes. Toenails were then rinsed repeatedly in Milli-Q water (Millipore Corp., Billerica, MA), dried, weighed, and digested in nitric acid (HNO_3; Trace Select Ultra Nitric Acid, Fisher Scientific International, Hampton, NH) at room temperature. The resultant solution was diluted to 8% HNO_3 and analyzed following the method described by Amarasiriwardena et al. (28) using an inductively coupled plasma-mass spectrometer (model 6100 DRC, Perkin-Elmer, Norwalk, CT).

Each sample was subjected to five replicate analyses. All samples were above the batch limit of detection (LOD), which ranged from 0.004 to 0.025 μg/g. Standard reference material water (NIST 1643d, Trace Elements in Water, National Institute of Standards and Technology, Gaithersburg, MD) and certified human hair reference material (CRM Hair; Shanghai Institute of Nuclear Research, Academia Sinica, China) were used to validate instrument performance and digestion method, respectively. The average percent recovery of NIST 1643d and CRM Hair was 92.9 ± 4% and 88.1 ± 7%, respectively.

The net concentration was calculated by subtracting detectable laboratory blank concentrations within each batch. The reported As concentrations were corrected for systemic error by normalizing the sample concentrations against the measured average daily NIST 1643d As_{in} concentration. This corrected value was used in all statistical analyses.

Drinking Water Collection and Analysis. Unfiltered drinking water was collected from the tube well each family identified as their primary drinking water source. For the first three sampling quarters, a single water sample was collected. For the remaining five sampling quarters, three water samples were collected on consecutive days to evaluate the possibility of short-term variability in tube well As concentrations.

Each tube well was purged for several minutes before sample collection. Water was then collected in acid-washed polyethylene containers and preserved with reagent-grade HNO_3 (Merck, Darmstadt, Germany) to a pH <2. Total inorganic As analysis was done by Environmental Laboratory Services (North Syracuse, New York) following U.S. Environmental Protection Agency method 200.8. The LOD for this method is 1 μg As/L. PlasmaCAL Multielement QC Standard 1 Solution (SCP Science, Montreal, Quebec, Canada) was used to validate analysis. The average As_{in} percent recovery was 97.2 ± 3.4%. As concentrations below the LOD were set to half the LOD.

Genotyping. Multiplex PCR amplifications were done from genomic DNA extracted from whole blood following the Puregene protocol (Gentra Systems, Minneapolis, MN). Genotyping of *GSTM1* and *GSTT1* followed the protocol described by Liu et al. (29). Homozygosity for the *GSTM1* and *GSTT1*-null [*GSTT1*(−)] allele was indicated by the presence of an internal control product in concurrence with the absence of a 230- and 480-bp fragment, respectively. Genotyping procedures were validated by randomly selecting 5% of the samples and subjecting them to repeat analysis. Two researchers independently reviewed all genotyping results until 100% concordance was achieved.

Statistical Analysis. Toenail As and drinking water As concentrations were positively skewed. Consequently, both toenail and drinking water concentrations were transformed to their common logarithms. Quarterly water samples were averaged when multiple measurements were available. Geometric means and SEs were calculated for toenail and drinking water concentrations. Participant characteristics were compared between villages using nonparametric tests. Linear mixed models with random family intercepts were used to determine the effect of drinking water As on each family's toenail As measurements.

To account for toenail growth, drinking water samples collected 3, 6, and 9 months before toenail collection were assumed to contribute different amounts of As to the pooled toenail sample. The relative contribution of water samples collected at these time points to toenail As was determined using maximum likelihood iteration within a generalized additive mixed model framework employing penalized regression splines. The fractional contributions of each of the three lagged water concentrations were combined into a single lagged variable, which effectively integrated 9 months of drinking water As exposure. A random intercept was included in the model to account for autocorrelation among repeated measures on a single subject. A random family intercept could not be included in the dose-response model because the random family intercept would absorb the effect of drinking water As because each member of a family used the same tube well. All statistical goals were achieved by estimating variables in the following nonlinear additive mixed model:

$$\log_{10} Y_q = \mathbf{x}^T\boldsymbol{\beta} + f\{\log_{10}(\mathbf{u}_q^T\boldsymbol{\gamma})\} + b + \varepsilon_q \qquad \text{(Eq. A)}$$

where Y_q represents an outcome collected in quarter q, x, represents a vector of time-independent covariates, $\boldsymbol{\beta}$ is a vector of linear regression coefficients, $\mathbf{u}_q$ represents a vector of three lagged drinking water As concentrations, $\boldsymbol{\gamma}$ is a vector of distributed lag coefficients, b represents a normally distributed random subject-specific intercept, ε_q represents a normal error, and f is a function of unknown form. Assuming $\boldsymbol{\gamma}$ is known, the model is a linear additive mixed model whose variables can be estimated using penalized likelihood or alternatively using maximum likelihood within a mixed model framework (30, 31).

Consequently, the vector of distributed lags was estimated by maximizing the likelihood for the corresponding linear additive mixed model over possible values of $\boldsymbol{\gamma}$. The nonparametric function f was represented using a basis of truncated linear functions whose knot locations were obtained from the unique

values of 30 quantiles of the weighted lag exposure $u_q^T\gamma$. Parametric bootstrap methodology with 500 resampled statistics was used to obtain SEs and bias-corrected accelerated (BAC) 95% confidence intervals (95% CI) for fitted regression variables and the weighed lag exposure variable (32).

A fully parametric linear mixed model was used to summarize the effect of drinking water As concentrations on toenail As content. To approximate the nonparametric effect estimated in Eq. A, a threshold value of 2 μg As/L (the maximum of lagged drinking water and 2 μg As/L) was used. Standard diagnostic techniques were used to validate distribution assumptions and evaluate covariate influence. The statistical program R version 2.0.1 was used for all analysis (33).

Results

Of the 248 participants comprising 50 families originally recruited for the repeated measures study, two families (n = 10) moved out of the area in January 2001 and were not included in this analysis. An additional family (n = 5) moved out of the area in September 2004, but their data were retained in the analysis because these individuals contributed to seven of the eight sampling quarters. Subsequently, 223, 217, 207, 209, 210, 199, 190, and 178 individuals contributed both a toenail and a water sample for quarters 1 to 8, respectively, reflecting an initial participation rate of 96% that declined to 76% in quarter 8. Quarterly mean drinking water As concentrations declined significantly over the course of the study from 99 μg As/L in quarter 1 to 64.8 μg As/L; however, this is most likely a result of seven families (n = 33) that used multiple tube wells over the course of the study. Of these seven families, five families (n = 24) switched from highly contaminated tube wells to As-free tube wells, whereas two families (n = 9) used multiple tube wells with different As concentrations.

Participants residing in the three recruitment villages were similar in age, gender, body mass index, smoking status, and betel nut chewing behavior. Each village also had a similar percentage of individuals possessing the *GSTT1*(−) genotype. Drinking water As concentrations, toenail As content, and individuals possessing the *GSTM1* genotype were found to vary significantly between villages (Table 1).

Drinking water concentrations ranged from <1 to 752 μg As/L (geometric mean, 6.2 μg As/L; SE, 2.7 μg As/L). Overall, 32% of the tube wells exceeded the Bangladesh drinking water standard of 50 μg As/L and 42% exceeded the WHO guideline for drinking water quality of 10 μg As/L. In the 41 tube wells that were used continuously by a single family, As concentrations were remarkably stable. Over half of these wells (53%) had a SD below 1 μg As/L, and 42% of the remaining tube wells had a SD between 1 and 10 μg As/L. Specifically, tube wells that had As concentrations below 10 μg As/L showed no variability in this 2-year period (Fig. 1A). Furthermore, no short-term variability was observed within the five sampling quarters in which water samples were collected on 3 consecutive days.

Toenail As concentrations ranged from 0.13 to 14.04 μg As/g (geometric mean, 1.01 μg As/g; SE, 0.04 μg As/g). Toenail As and drinking water As were highly correlated with a Spearman correlation coefficient of 0.73 (P < 0.0001). The distribution of toenail As concentrations for each family is illustrated in Fig. 1B. Toenail As concentrations in individuals who used drinking water with As concentrations below 1 μg As/L and could therefore be considered unexposed to contaminated drinking water ranged from 0.13 to 6.69 μg As/g (geometric mean, 0.64 μg As/g; SE, 0.03 μg As/g). Toenail As concentrations were higher for individuals' with drinking water >1 μg As/L (geometric mean, 1.32 μg As/g; SE, 0.05 μg As/g; range, 0.14-14.04 μg As/g).

The relationship between drinking water As and toenail As was observed to be nonlinear (Fig. 2). Age was found to modify the effect of drinking water As on toenail As (β = 0.01; 95% CI, 0.0001-0.02). *GSTT1*(−) was also significantly associated with toenail As (β = 0.08; 95% CI, −0.001 to 0.15; Table 2). Other physical and sociodemographic predictors [i.e., gender, body mass index, smoking, betel nut usage, and *GSTM1*(−)] were not associated with the outcome. The best-fitting parsimonious model was as follows:

$$\log_{10} Y_{iq} = \beta_0 + \beta_{\text{age}}\text{age}_i + \beta_{\text{null}}\text{GSTT1}_i + \beta_{\text{age}}\text{age}_i \times \max\{\log_{10}(2.0), \text{weighted}_{iq}\} + f(\text{weighted}_{iq}) + b_i + \varepsilon_{iq}, \quad \text{(Model 1)}$$

where $\text{weighted}_{iq} = \log_{10}(\Sigma_{l=l}^{3}\gamma_l \text{water}_{iql})$.

The notation for Model 1 is described in Eq. A. Briefly, Y_{iq} is the toenail As detected in the ith individual for sampling quarter q, age is centered based on the median participant age of 27 years at recruitment, and genotype is *GSTT1*(−). In this model, the weights γ_l were determined to be 0.70 (95% CI, 0.39-0.90), 0.14 (95% CI, 0.003-0.34), and 0.16 (95% CI, 0.04-0.39) for water collected 3, 6, and 9 months before toenail

Table 1. Summary statistics, including percentile, mean (SD), geometric mean (SE), and range of physical, social, and demographic characteristics of participants residing in the three recruitment villages

Characteristics	Village 6 (n = 90)	Village 5 (n = 16)	Village 12 (n = 129)
Physical			
Gender (%)			
Male	43	50	45
Female	57	50	55
Mean (SD) age, range (years)	32.2 (15.3), 10-70	29.8 (17.9), 10-77	29.2 (14.2), 7-70
Mean (SD) body mass index, range	19.9 (3.6), 12-27	20.9 (4.3), 13-30	19.7 (3.7), 14-29
GSTM1(−) genotype (%)*	28.8	35.7	62.5
GSTT1(−) genotype (%)	18.9	6.3	19.4
Social			
Smokers in same environment (%)	22.2	18.8	16.3
Chewing betel nuts (%)	38.2	6.8	54.9
Demographic			
Geometric mean (SE) drinking water As, range (μg/L)	55.2 (35.4),† ND-752	42.4 (7.4),† ND-138	1.17 (1.8),† ND-32.5
Geometric mean (SE) toenail As, range (μg/g)	0.267 (0.007),† 0.034-14.04	0.988 (0.083),† 0.34-4.87	0.66 (0.020),† 0.13-8.97
Mean (SD) duration of tube well use, range (years)	9.1 (7.0), 1-20	9.6 (7.2), 2-20	8.6 (4.7), 2-19
Mean (SD) water volume consumed daily, range (L)	3.4 (0.8), 2-5	3.2 (0.9), 2-5	3.1 (0.9), 1-5

*P = 0.03.
†P < 0.0001.

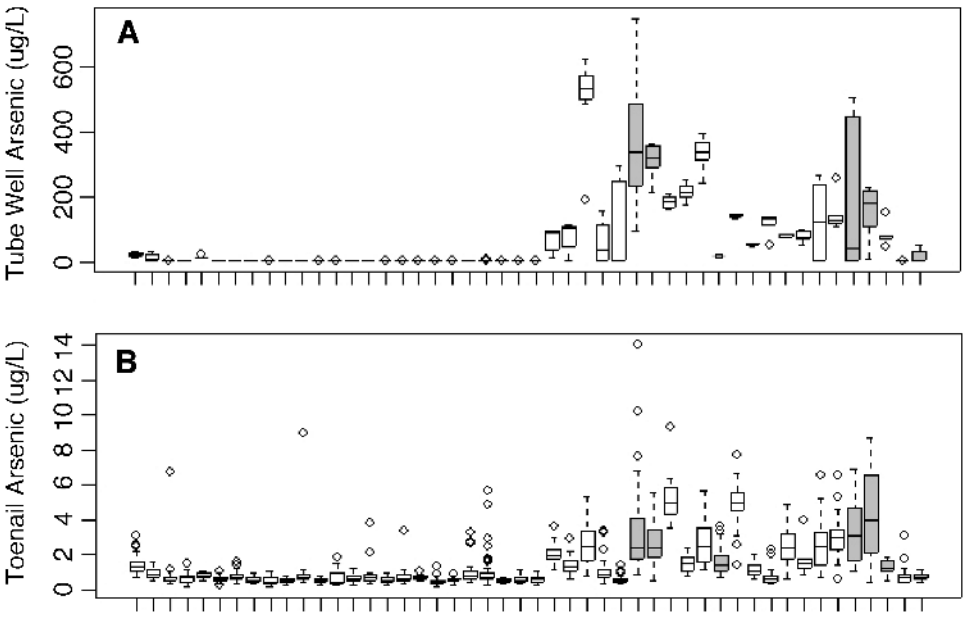

Figure 1. Descriptive illustration of tube well As (**A**) and toenail As concentrations (**B**) within each family. *Shaded box plots,* families that used multiple wells during this study.

collection, respectively. The correlation between these weight variables and *GSTT1*(−) were <0.08, indicating that the estimates generated for *GSTT1*(−) are reasonably independent of the weighting scheme employed. The residual variance was 0.143 and the within-subject variance was 0.208. Thus, the intrasubject correlation was 0.68 ($P < 0.0001$), indicating that toenail As concentrations were fairly stable within an individual.

Visual inspection of the dose-response curve generated by Model 1 revealed no apparent relationship between drinking water As exposure and toenail As content when drinking water concentrations were <2 μg As/L (Fig. 2). Above 2 μg As/L, toenail As concentrations increased in a dose-dependent manner with increased drinking water As concentrations. Above 100 μg As/L, the slope of the regression line between drinking water As and toenail As seemed to decline. However, this was within the SEs generated by the bootstrap models.

The estimates of the predictors used in Model 1 are described in Table 2. For subjects at the median age (27 years), a 10-fold increase in drinking water As above 2 μg As/L resulted in ~1.6-fold increase in toenail As. A positive interaction between age and drinking water As was observed with each 5-year increase in age multiplying this effect by 59%.

It was not possible to evaluate how familial association influenced toenail As because each member of a family used the same tube well; thus, random family intercepts would absorb the effect of As exposure. A large portion of the interfamily variability was explained by the variability in drinking water As (Fig. 3). We therefore believed that the best model should use drinking water As to generate a dose-response relationship.

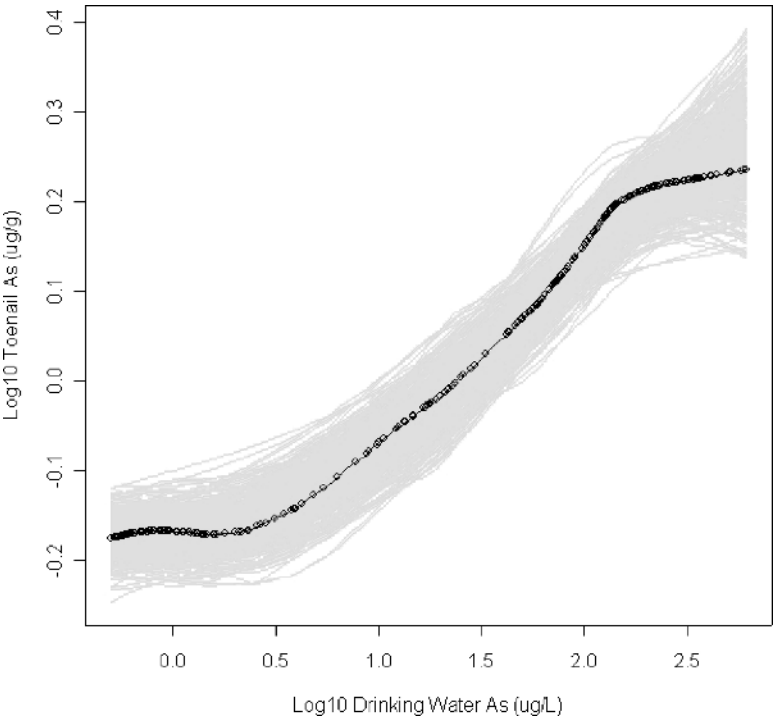

Figure 2. Model 1 fitted regression line for participant with median age of 27 years using a weighed lagged drinking water exposure that attributed 70% (95% CI, 0.39-0.90), 14% (95% CI, 0.003-0.34), and 16% (95% CI, 0.04-0.39) to water samples collected 3, 6, and 9 months before each toenail collection. 95% CIs are indicated by 500 resampled statistics.

Table 2. Results of regression models with the outcome $\log_{10}$ toenail As (μg As/g) for a participant of median age (27 years)

Predictors	Estimate	SE*	95% CI†	*P*
Model 1: Fixed effects				
Intercept	−0.155	0.047	−0.183 to −0.089	<0.0001
Age‡	−0.005	0.005	−0.02 to 0.01	0.38
GSTT1(−)	0.076	0.036	−0.001 to 0.154	0.03
Age‡ × $\log_{10}$ drinking water As (μg/L)	0.010	0.004	0.0001 to 0.02	0.02
$\log_{10}$ drinking water As (μg/L)§	0.191	0.012	—	<0.0001
Model 2: Gene-environment interaction				
Intercept	−0.135	0.051	−0.169 to 0.058	<0.001
Age‡	−0.010	0.005	−0.025 to 0.005	0.28
GSTT1(−)	−0.064	0.053	−0.181 to 0.036	0.41
Age‡ × $\log_{10}$ drinking water As (μg/L)	0.010	0.005	0.002 to 0.02	0.02
GSTT1(−) × $\log_{10}$ drinking water As (μg/L)	0.125	0.035	0.064-0.2	0.001
$\log_{10}$ drinking water As (μg/L)§	0.177	0.013	—	<0.0001

NOTE: The estimates for drinking water concentrations are valid above 2 μg As/L.
*SEs estimated via bootstrap (500 resampled statistics).
† Nonparametric bootstrap bias-corrected accelerated 95% CIs.
‡ Intercept is for median age of 27 years and the estimate is for 5-year increments.
§Estimates of drinking water effect are based on fully parametric model approximating the nonparametric effect depicted in Figs. 2 and 4 for drinking water above 2 μg As/L.

Based on the results of Model 1, a multiplicative interaction between *GSTT1*(−) and drinking water As was investigated. To avoid the loss of power that would come from estimating a second spline term, we used a parametric threshold term similar to the one used to summarize the slope value. The multiplicative interaction term can therefore only be interpreted for drinking water As concentrations above 2 μg As/L. This relationship was described by the following:

$$\log_{10} Y_{iq} = \beta_0 + \beta_{\text{age}}\text{age}_i + \beta_{\text{null}}\text{GSTT1}_i + \beta_{\text{age}}\text{age}_i \times \max\{\log_{10}(2.0), \text{weighted}_{iq}\} + \beta_{\text{null}}\text{GSTT1}_i \times \max\{\log_{10}(2.0), \text{weighted}_{iq}\} + f(\text{weighted}_{iq}) + b_i + \varepsilon_{iq} \quad \text{(Model 2)}$$

where $\text{weighted}_{iq} = \log_{10}(\Sigma_{l=1}^{3}\gamma_l \text{water}_{iql})$.

The BAC 95% CIs generated from the bootstrap analysis with 500 replicates for the three water samples integrated into the weighted lag exposure variable were very similar to those calculated in Model 1, with 69%, 14%, and 17% of the exposure attributed to drinking water As concentrations measured 3, 6, and 9 months before each toenail collection (95% CI 0.46-0.97; 0.001-0.31; 0.03-0.35). The correlation between these weight variables and *GSTT1* was 0.09, similar to those calculated for Model 1, supporting the conclusions that the weighting scheme employed to capture drinking water As concentrations that occurred during toenail formation did not greatly influence the effect estimate of *GSTT1*(−).

The influence of assigning a 2 μg As/L threshold for the parametric term was evaluated by systematically substituting thresholds ranging from 1 to 3 μg As/L. However, the choice did not affect the estimates. The residual variance, within-subject variance, and intrasubject correlation was similar to those reported in Model 1 (0.143, 0.200, and 0.66, respectively).

Model 2 found that, for every 5-year increase in age, toenail As increased by 47% (95% CI, 0.002-0.02), which was very similar to the effect observed in Model 1 (Fig. 4). A significant interaction between *GSTT1*(−) and drinking water As concentrations on toenail As content was observed. For every log unit increase in drinking water As exposure above 2 μg As/L, median aged participants with a *GSTT1* wild-type genotype had a 1.5-fold increase in toenail As content, whereas participants with *GSTT1*(−) genotypes had a 2-fold increase in toenail As content (95% CI, 0.064-0.2) (Table 2).

Discussion

Toenails are an integrated measure of As exposure and a biological marker of internal dose. To accurately characterize the relationship between exposure and toenail As content, it is important to capture the physiologically relevant As exposure that occurred during nail formation. Without growth rate measurements, it was necessary to extrapolate As exposure from drinking water samples collected before the toenail collection. This relationship was complicated by methodologic requirements necessitating nail collection from all toes to ensure sufficient mass for detection purposes. Depending on the nails sampled, this mass could reflect exposures that occurred several months up to 1 year before nail collection.

The number of individuals who contributed both a toenail sample and a drinking water sample declined over the course of the study from 96% to 76%. This is an excellent

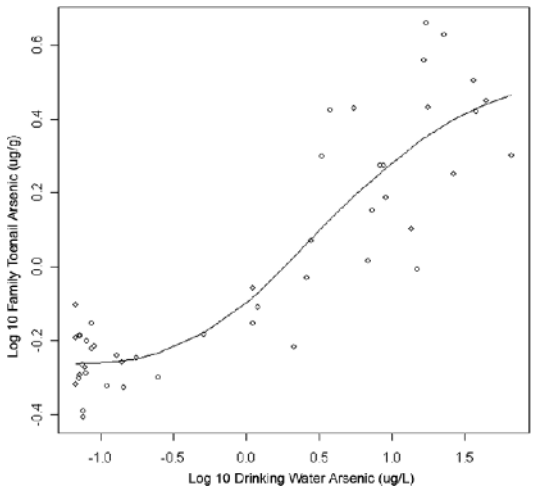

Figure 3. Illustration of the effect of drinking water As on a family's toenail As concentration.

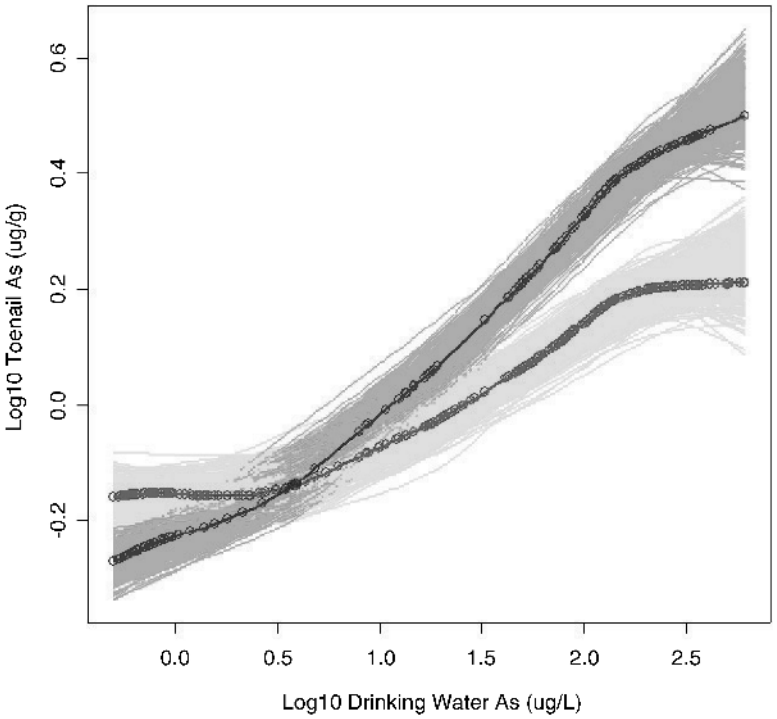

Figure 4. Fitted regression line for Model 2 illustrating the gene-environment interaction for *GSTT1*(−) for participant with median age of 27 years. 95% CIs are indicated by 500 replicates from larger bootstrap model. Drinking water exposure is an integrated measure of 9 months of drinking water As.

participation rate for a repeated-measures study of this duration. Scrutiny of collection records indicated that samples were missing completely at random, which is further supported by the fact that no participant requested to be withdrawn from the study, although participants were aware of this option. This leads us to believe that the missing samples are a result of our collection strategy where members of the field team visited each home several times each quarter to collect samples. However, if a participant was not at home on those days, no samples were collected.

Repeated measurements taken from individual tube wells found no short-term within-quarter variation for the five sampling quarters that collected water samples on 3 consecutive days. Furthermore, the majority of the tube wells that were continuously monitored had stable As concentrations. The fluctuations that were observed could be natural variability in the groundwater. Researchers have found that dissolved As concentrations are maximized at a depth of 37 to 39 m in the aquifer that underlies the Bengal basin (34). If the depth of the aquifer changed, this As-enriched zone could migrate in relation to the stationary tube wells. However, we did not find an association between tube well depth and As concentration or variability. Another explanation for the observed variability is that unfiltered water samples were collected and the preservation process, which involved acidifying the samples, could have resulted in the dissolution of particulate-bound As.

The weighted lagged exposure variable created in this study included drinking water As concentrations measured 3, 6, and 9 months before toenail collection in which each time period contributed 69%, 14%, and 17% to the As detected in the pooled toenail sample (95% CI, 0.46-0.97, 0.001-0.31, and 0.03-0.35, respectively). This seemed to suggest that drinking water exposure that occurred 3 months before toenail collection contributed more As to the nail than earlier exposures. The 95% CIs on the percent contribution of each weighted lag are large. This could be a result of toenail material that was lost to collection either as a result of personal grooming or breakage between sampling visits as well as difference in toenail growth rates. It could also reflect misclassification of exposure because it is likely that participants used more than one drinking water source in this 2-year period. The misclassification would be more substantial for participants with highly contaminated tube wells if they occasionally sought out safer drinking water.

The weighted lagged exposure variable integrated 9 months of drinking water As concentrations. This time interval is similar to those reported by Longnecker et al. who observed that toenail selenium concentrations, among individuals provided with selenium-enriched bread, increased in great nails after 24 weeks and in all other nails after 12 weeks. Selenium concentrations reached a steady state after 26 weeks in all nails, except the great nails, which never did achieve steady state even after 1 year of increased dietary selenium intake (20). Thus, it is possible that the clippings from the great nail, which are included in our pooled samples, reflect exposures that occurred over 12 months before the nail being collected.

We investigated incorporating a fourth drinking water sample that was collected 12 months before the toenail sample collection in the weighted lagged exposure variable but found that the contribution from this time point was negligible to toenail As. Therefore, we did not include this additional water sample in our models because each lag included in the weighted exposure variable resulted in a loss of data and hence power.

Failing to properly account for toenail growth would result in inaccurate exposure estimates and bias our results. Specifically, it would overestimate the effect of drinking water As among those individuals who minimized their exposure to As-contaminated drinking water if their nail measurements contained As from exposures that occurred >9 months previously. Of the 48 families who participated in this study, 5 families (n = 24) switched from highly contaminated tube wells to As-free drinking water. The toenails collected from these individuals were the most valuable in determining the appropriated weighted lagged exposure because their toenail samples formed a matched set of observations that allowed for the direct observation of the effect of changing As exposure, ultimately reducing the variability in our estimates. Therefore, we feel confident that the 9 months of drinking water exposure used in our models was of sufficient duration to capture a physiologically relevant dose. However, future researchers

using toenails, or other biomarkers that reflect prior exposure history, should measure growth rates to prevent exposure misclassification.

The dose-effect relationship between toenail and tube well As concentrations was modeled using semiparametric techniques, which make very weak assumptions regarding the mathematical form of the relationship. We believe that the linear dose-response relationship observed above the threshold of 2 μg/L is generalizable to other populations. However, the baseline concentrations (i.e., intercept and concentrations below the threshold) reported by our models would vary depending on the population, as these values would be highly dependent on background concentrations.

A study in New Hampshire that looked at the relationship between drinking water exposure and toenail As concentrations found that for every 10-fold increase in drinking water As concentrations, toenail As increases 2-fold (35). They also observed that the correlation between drinking water As and toenail was very strong (ρ = 0.65) when drinking water concentrations were >1 μg As/L but became insignificant (ρ = 0.08) for drinking water concentrations below 1 μg As/L (36). Our results supported these findings because we observed a 1.6-fold increase in toenail As concentrations in our overall population and a 2-fold increase among *GSTT1*(−) genotypes, with every 10-fold increase in drinking water As above 2 μg As/L. We also observed that toenail As and drinking water As were not associated when drinking water As concentrations were <2 μg As/L.

Toenail As has been observed to correlated with dietary As intake (37). Surveys of individual food items in Bangladesh have found that rice and vegetables, staples in the Bangladesh diet, contain elevated As concentrations (38). Thus, dietary As intake could become the dominant route of exposure when drinking water concentrations are very low and could explain why we did not observe a relationship between toenail As and drinking water As below 2 μg As/L (36). Therefore, it is possible that background exposure is greater in Bangladesh; however, a more thorough exposure assessment would be needed to verify this observation and determine if dietary As intake is responsible for the observed threshold and whether it would influence the observed dose-effect relationship.

It is also possible that nutritional status could influence As metabolism and change the distribution of As in tissues. In our study, 35% of the participants had a body mass index below 18, indicating that they were underweight and possibly malnourished. However, very few studies have investigated the association between diet and As metabolism. One animal study found that rabbits fed low-protein diets have decreased urinary excretion of dimethylarsenic acid (DMA), a metabolite of inorganic As, and higher tissue As concentrations (39). Additionally, a study in Taiwan that investigated As methylation capacity among 115 As-exposed individuals and biomarker response found that the percentage of DMA excreted in urine was positively associated with toenail As (22). Therefore, if protein deficiency or malnutrition could influence As metabolism, it is possible that it could influence the distribution of As in the body; however, further research is needed to evaluate the role of diet on As metabolism and biomarker response.

Age was found to positively modify the effect of drinking water exposure on toenail As. It has been suggested that toenail growth decreases with age, which could explain the observed relationship (40). In addition, this study recruited families, which included 53 children ages <15 years, who might have different metabolic processes that are influencing this interaction.

The significant multiplicative interaction between drinking water As exposure and toenail As content suggested that *GSTT1* is important for removing As_{in} from the circulatory system before As can bind to the sulfhydryl groups in keratin cells. The Taiwan study described previously did not find a direct association between *GSTT1* genotypes and toenail As but did find that among *GSTT1*(−) individuals the percentage of monomethylarsonic acid (MMA) in urine was negatively associated with toenail As, whereas the percentage of DMA in urine was positively associated with toenail As (22). This differs from our findings in which *GSTT1*(−) genotypes had significantly more toenail As compared with *GSTT1* wild-type. However, our study was twice as large as the Taiwan study. Therefore, it seems that individual difference in As metabolism could influence toenail As concentrations. It is important to further investigate the relationship between the GSTT1 gene and As metabolism in a larger study and using additional biomarkers, including urinary As metabolites, to more accurately assess whether individuals who lack the ability to produce GSTT1 metabolize As differently or possibly are more susceptible to As toxicity.

Toenails have been shown to have a high degree of reproducibility over time, suggesting that the biomarker has a low degree of within-person variability (21). We also found that an individual's toenail As concentrations were remarkably stable over time, a desirably characteristic for a biomarker that can validate exposure assessment. Toenails are a good biomarker of As exposure and offer several practical advantages, including the fact that they can be collected non-invasively and without specialized equipment, can be stored at room temperature, and are lightweight and easy to transport. However, their ability to differentiate exposure concentrations from drinking water sources may be limited at very low environmental concentrations.

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