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# Arsenic exposure and DNA methylation among elderly men

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## Abstract

**BACKGROUND—**Arsenic exposure has been linked to epigenetic modifications such as DNA methylation in in vitro and animal studies. This association has also been explored in highly exposed human populations, but studies among populations environmentally exposed to low arsenic levels are lacking.

**METHODS—**We evaluated the association between exposure to arsenic, measured in toenails, and blood DNA methylation in Alu and Long Interspersed Nucleotide Element-1 (LINE-1) repetitive elements in elderly men environmentally exposed to low levels of arsenic. We also explored potential effect modification by plasma folate, cobalamin (vitamin $B_{12}$), and pyridoxine (vitamin $B_6$). The study population was 581 participants from the Normative Aging Study in Boston, of whom 434, 140, and 7 had 1, 2, and 3 visits, respectively, between 1999-2002 and 2006-2007. We used mixed-effects models and included interaction terms to assess potential effect modification by nutritional factors.

**RESULTS—**There was a trend of increasing Alu and decreasing LINE-1 DNA methylation as arsenic exposure increased. In subjects with plasma folate below the median (< 14.1 ng/ml), arsenic was positively associated with Alu DNA methylation (β=0.08 [95% confidence interval = 0.03 to 0.13] for one interquartile range [0.06μg/g] increase in arsenic) while a negative association was observed in subjects with plasma folate above the median (β=-0.08 [-0.17 to 0.01]).

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**CONCLUSIONS—**We found an association between arsenic exposure and DNA methylation in Alu repetitive elements that varied by folate level. This suggests a potential role for nutritional factors in arsenic toxicity.

Arsenic is a ubiquitous environmental contaminant and the number-one chemical on the Environmental Protection Agency's CERCLA Priority List of Hazardous Substances.[1] Arsenic has been associated with increased risk of cancer,[2] cardiovascular disease,[3] and neurologic deficits,[4] although the mechanisms through which it acts are likely diverse. One potential pathway is through epigenetic changes, in that arsenic affects methylation metabolism and also is an oxidant.[4-6] Both properties are thought to influence DNA methylation.[7-9] DNA methylation, the most well-studied epigenetic mechanism, involves the addition of methyl-groups on cytosines to form 5-methyl cytosine (5-mC), which can repress gene expression due to a closed chromatin structure. DNA methylation also plays an important role in maintaining genome integrity by silencing the transcription of repetitive DNA sequences and endogenous transposons.[10] A large proportion of the human genome is composed of Class I transposons and retrotransposons (collectively referred to as “repetitive elements”), which are viral DNA remnants that can move to different positions within the genome of a single cell. The most abundant families of retrotransposons are Alu and LINE-1, which represent approximately 30% of the human DNA.[11,12] Hypomethylation of these otherwise heavily methylated elements[13] can enhance their activity as retrotransposons, which can in turn adversely affect the normal function of cells by inserting mutations[14] or introducing genomic instability.[10] Epigenetic modifications in Alu and LINE-1 elements have been associated with aging and with various risk factors for the same diseases associated with arsenic, such as cancer, cardiovascular and neurologic diseases.[15-18]

Global and gene-specific methylation changes have been linked to arsenic in in vitro, animal and human studies.[5,6,19-26] Two studies have examined the association of arsenic with global DNA methylation in humans, and both found a positive association.[5,23] Arsenic consumes methyl groups provided by the main methyl donor, intracellular S-adenosylmethionine (SAM). If critical dietary sources such as folate are relatively low, this may result in hypomethylation of competing substrates such as DNA. Folate is needed to methylate homocysteine to form methionine, the precursor of SAM, a reaction that is catalyzed by a vitamin $B_{12}$-containing methyltransferase. Therefore the effects of arsenic might be modified by the availability of methyl donors and one-carbon metabolism factors such as vitamins $B_{12}$ and $B_6$.[5] However, the few published human studies have focused primarily on highly exposed populations, in which the impact of dietary factors on arsenic metabolism and DNA methylation would likely differ substantially from persons with lower arsenic exposure. To our knowledge no studies are available among people exposed to low arsenic levels.

We hypothesized that As exposure was associated with decreased DNA Alu and LINE-1 methylation. We examined this association in a population of environmentally exposed elderly men and explored potential modification by plasma folate, $B_{12}$, and $B_6$.

## METHODS

### Study population

The study population originated from the Normative Aging Study, a cohort of community-dwelling men in eastern Massachusetts that has been followed by the Veterans Administration since 1963.[27] Participants visit the Veterans Affair Outpatient Clinic in Boston for comprehensive clinical examinations and standard laboratory tests every 3-5 years. Prior to each scheduled visit, participants are asked to collect and bring their toenail

clippings. The study visits occurred early in the morning after fasting overnight and abstaining from smoking. The annual attrition rate has been approximately 1%.

Our study period extended from 1 March 1999 to 12 November 2002 and from 10 May 2006 to 7 November 2007 (toenail samples were not collected between 13 November 2002 and 9 May 2006 due to a hiatus in grant funding). Of the 767 participants who had at least one follow-up visit during the study period, 744 (97%) had at least one available DNA methylation measurement in either Alu or LINE-1 from blood DNA, and 735 had both Alu and LINE-1 measurements. DNA methylation was quantified in every blood sample at three Cytosine-phosphate-Guanine (CpG) dinucleotide loci. Among these participants, 594 contributed a toenail sample. We excluded participants' visits with invalid DNA methylation (1 visit), invalid toenail arsenic measurement (1 visit), toenail arsenic levels below the detection limit (3 visits), or missing data on relevant covariates (14 visits). This resulted in the exclusion of 13 subjects and 19 visits. Final analyses were performed on 581 participants, of whom 434, 140, and 7 had 1, 2, and 3 visits, respectively, for a total of 735 visits and 2205 DNA methylation measurements.

The study was approved by the Institutional Review Boards of all participating institutions. All participants gave written informed consent before the study.

### Toenail sample analysis

Study participants collected toenail clippings from all 10 toes and brought them to each visit. After cleaning, toenail samples were analyzed by inductively coupled plasma mass spectrometry (Elan 6100, Perkin Elmer, Norwark, CT), as detailed in the eAppendix 1 (http://links.lww.com). We measured each sample five times and used the average as the analytic value.

### DNA methylation analysis

Seven-ml blood samples were collected in EDTA tubes during each study visit. Buffy coat was extracted and stored in a cell lyses solution until DNA extraction. All samples were coded and frozen at –20°C. Samples were sent to the laboratory in five batches. DNA was extracted using QiAmp DNA blood kits (QIAGEN, Hilden, Germany). The samples were bisulfite-treated using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA). Repetitive elements DNA methylation was quantified by PCR and pyrosequencing using the Pyromark MD System (Pyrosequencing Inc., Westborough, MA). Pyrosequencing was performed using previously described methods for analyzing the methylation of repetitive elements[13] with minor modifications described elsewhere.[16] Each sample was pyrosequenced in duplicates.

The degree of methylation in both Alu and LINE-1 repetitive elements was measured at each of three CpG dinucleotide loci that are repeated over the human genome with the sequence of interest. Methylation was expressed as the percentage of methylated cytosines (%5mC) over the sum of methylated and unmethylated cytosines. To increase the precision of our results, we used all three CpG loci measurements in the statistical analysis. The within-sample coefficients of variation (CVs) were 3.47, 4.04 and 5.35 for the first, second and third Alu locus, respectively, and 1.58, 1.36 and 1.91 for the first, second and third LINE-1 locus, respectively. Each plate included controls containing water, annealing solution, binding solution, and PCR product.

### Folate and vitamins $B_{12}$ and $B_6$ analysis

At each visit, plasma folate and vitamin $B_{12}$ concentrations were measured by radioassay with the use of a commercially available kit from Bio-Rad (Hercules, CA), and plasma

vitamin $B_6$ concentration was measured enzymatically. The CVs for the folate, $B_{12}$, and $B_6$ assays were 4.3%, 4.7%, and 5.0%, respectively. Further details are found elsewhere.[28]

## Covariate assessment

At each study visit, extensive physical examination, anthropometric, questionnaire and laboratory data were collected. Height and weight measurements were included in the physical examination, and body mass index (BMI) was calculated as weight(kg)/height(m)$^2$. A self-administered questionnaire was employed to collect health- and lifestyle-related information, including medical history and cigarette smoking. Medical information was confirmed by an on-site physician. Alcohol consumption was determined by a standardized semi-quantitative food frequency questionnaire.[29]

## Statistical analysis

Participants were compared with non-participants at their first visit during the study period. We used t tests to compare two groups of normally distributed continuous variables, while the Wilcoxon rank-sum test was used to compare two groups of skewed variables. Chi-square or Fisher's exact tests were used to test for group differences in the categorical variables. Pearson correlation coefficient (r) was used to calculate the correlation between two normally distributed variables. We calculated the intraclass correlation coefficients (ICCs) for Alu and LINE-1 DNA methylation by running a mixed-effects model with a random effect for each participant.

We evaluated the association between toenail arsenic concentration and repetitive elements DNA methylation measured in blood samples collected at the same visit when toenail samples were brought in. Separate models for Alu and LINE-1 DNA methylation were fitted. DNA methylation was analyzed at three separate CpG dinucleotide loci for each subject. Linear mixed-effects models were used to account for the correlation among repeated measurements within the same subject. These included a random intercept for each subject in order to account for the heterogeneity in their overall level of methylation.

We considered possible confounders or important predictors of the outcome—age, cigarette smoking status, pack-years, BMI, alcohol consumption status, percent lymphocytes in differential leukocyte counts, season and day of the week of the visit, and laboratory batch for the DNA blood samples—on the basis of their biologic significance and information from previous studies.[5,16,30,31] Age, laboratory batch and CpG dinucleotide locus number were a priori included in the models, while the rest of the covariates were added based on whether their addition to the model significantly changed the effect estimate or improved the model's fit as evaluated by a likelihood ratio test. The final models included age, laboratory batch, CpG dinucleotide locus number, percent lymphocytes, BMI, and alcohol drinking status. The structure of the fitted models is provided in eAppendix 2 (http://links.lww.com).

To examine possible non-linear associations between arsenic and DNA methylation levels, we modeled toenail arsenic as a penalized spline by using generalized additive models. The penalized spline is a cubic regression spline with 10 knots; however, the coefficients of the spline are penalized, which constrains the number of degrees of freedom used.[32] We used multiple generalized cross-validation to choose the degree of penalty. With this method, the data are divided into subsets, and after omitting each subset in turn, the model is successively fit. The fitted model is subsequently used to “predict” the response for the subset that was left out. This procedure is repeated with different smoothing parameter values, which suggests a value that minimizes the cross-validation estimate of the mean-squared error.[33] The model fit was also examined by identifying outliers and other highly influential data values by means of graphical and visual inspection and calculation of

influence diagnostics. The list of the diagnostics is included in eAppendix 3 (http://links.lww.com).

To explore potential effect modification of the main associations by plasma folate, vitamin $B_{12}$ and vitamin $B_6$, we included an interaction term between toenail arsenic and each of the nutritional factor variables in our main-effects models. The nutritional-factor variables were dichotomized at the median.

All mixed-effects models and influence diagnostics were conducted using SAS (version 9.2 SAS Institute Inc., Cary, NC) while the generalized additive models were run in R (version 2.10.1, R Foundation of Statistical Computing, www.r-project.org).

## RESULTS

No meaningful differences were found between participants (n=581) and non-participants (n=186) at the baseline visit in terms of age, BMI, smoking or drinking status, percent lymphocytes, and average Alu and LINE-1 DNA methylation (data not shown). The ICCs for Alu and LINE-1 methylation were 0.01 and 0.34, respectively, indicating that there is intraindividual variability in Alu methylation with time, while LINE-1 methylation remains rather stable.

At the baseline visit, study participants had a mean age of 72 years (SD=7). The majority were former smokers (67%) and had consumed fewer than 2 drinks per day in the year preceding the visit (81%) (Table 1). DNA methylation in Alu elements was weakly and inversely correlated (r=-0.2) with DNA methylation in LINE-1 elements.

In the generalized additive models, the generalized cross-validation estimated one degree of freedom when fitting a penalized spline for toenail arsenic in both LINE-1 and Alu DNA methylation models. We therefore concluded that modeling the exposure of interest as a linear term in our models was appropriate.

In the final covariate-adjusted linear mixed effects models, arsenic levels showed a trend of negative association with LINE-1 methylation ($\beta$=-0.05 per one interquartile range [IQR=0.06μg/g] increase in toenail arsenic [95% confidence interval = -0.11 to 0.02]), while a trend of positive association was observed between arsenic and Alu methylation ($\beta$=0.03 [-0.01 to 0.07]). Further adjusting for smoking status, pack-years, season and day of the week did not change the estimates (Table 2).

When we examined the main associations in visits with complete data for plasma folate and vitamins $B_{12}$ and $B_6$ from 547 participants, the associations retained the same directions (for LINE-1, $\beta$=-0.03 [95% CI= -0.11 to 0.03]; for Alu, 0.04[-0.004 to 0.083]).

Results from the models including the interaction terms are found in Table 3. The association between arsenic and Alu methylation was modified by plasma folate status. For a one-IQR (0.06μg/g) increase in toenail arsenic, Alu methylation decreased by 0.08% for participants with plasma folate above the median (>14.1 ng/ml), while for participants with plasma folate below the median, an increase of 0.08% in Alu methylation was observed for the same increment of toenail arsenic. We observed no difference in the association between arsenic and LINE-1 methylation in men with plasma folate below or above the median, or in the association between arsenic and Alu or LINE-1 in men with plasma vitamins $B_{12}$ or $B_6$ above and below the median (Figure).

## DISCUSSION

In this cohort of elderly men environmentally exposed to low arsenic levels, LINE-1 DNA methylation tended to decrease, while, contrary to our hypothesis, Alu DNA methylation tended to increase with increasing arsenic (although these associations were weak). Methylation decreased with age. For a one-year increase in age, Alu and LINE-1 DNA methylation decreased by 0.01% (95% CI = -0.03 to -0.00) and 0.02% (-0.04 to -0.00) respectively. By comparison, the associations with arsenic were stronger for LINE-1 (β=-0.05 [95% CI= -0.11 to 0.02) and in the other direction for Alu (β=0.03[-0.01 to 0.07]). Similarly the association of percent lymphocytes was in the expected direction (for 1% increase in percent lymphocytes, LINE-1 and Alu DNA methylation decreased by 0.02% [-0.04% to -0.01%] and 0.01% [-0.02% to -0.00%], respectively).[31] Stronger negative associations between other environmental exposures (such as lead, $SO_4$, $PM_{2.5}$ and black carbon) and LINE-1 methylation have also been found among participants in this study.[30,34,35] When we examined potential confounding of our associations of interest by lead and particulate air pollution, no confounding was detected (eAppendix 4, http://links.lww.com).

Folate nutritional status modified the association between arsenic and Alu DNA methylation. Men with lower values of plasma folate showed an increase in Alu DNA methylation with increasing arsenic, while a decrease of the same magnitude was observed among men with higher values of plasma folate. No differences in the associations of arsenic with LINE-1 DNA methylation were observed in people with lower or higher values of B vitamins. The same was true for the association between arsenic and Alu methylation in men with lower or higher values of plasma vitamins $B_{12}$ and $B_6$. Nonetheless, all the above associations appeared stronger among men with lower values of plasma folate and vitamins $B_{12}$, and $B_6$, indicating a possible role of vitamin B nutrients in the arsenic mechanism of action.

One mechanism through which arsenic may be associated with DNA methylation is by interference with methyl donor availability.[7] Methyl groups provided by the methyl donor S-adenosylmethionine are consumed during the metabolism of arsenic, which can lead to global DNA hypomethylation. However, this possibility is unlikely in our study population, given low arsenic levels and high folate values (median plasma folate=30nmol/L at baseline visit). Hypomethylation can also occur as a result of oxidative DNA damage. Arsenic and metals in general increase the production of reactive oxygen species (ROS) via redox cycling,[5,6] and long-term exposure to oxidative stress has been associated with oxidative damage of methylated cytosine residues and gradual loss of cytosine methylation in repeated elements.[8,9] However, it is unlikely that the low levels of arsenic exposure in our study population would yield sufficient oxidative stress to induce measurable changes in DNA methylation – which may be why our observed associations were not stronger.

In vitro and animal studies have found an inverse relation between exposure to arsenic and genomic DNA methylation as measured in hepatic cells.[7,25] In human populations, arsenic has been linked with hypermethylation of tumor-suppressor genes in cancer[19,26] and arsenicosis[21,22] patients. In a population chronically exposed to arsenic-contaminated water in Bangladesh, arsenic exposure was associated with increased genomic blood leukocyte DNA methylation.[5] Our observation of a trend of positive association between arsenic and Alu DNA methylation seems to be consistent with this finding, but the two studies differ in several aspects. We used toenails, a measure of longer-term arsenic exposure than blood, urine or plasma (used by Pilsner et al.[5]), which reflect more recent exposures. Furthermore, the average folate levels in our study were nearly three times higher (with a median at baseline of 13.3ng/ml, or 30nmol/L) than those reported in Pilsner et al. (mean of 9nmol/L).

This discrepancy could also explain some of the differences in effect modification by plasma folate between the two studies. We found that men with "low" plasma folate levels (≤14.1ng/ml or equivalently ≤32nmol/L) showed an increase in Alu DNA methylation with increasing arsenic. These men would be somewhat comparable to those in the Bangladesh study[5] who were assigned to the "high" folate group (>9nmol/L or >4ng/ml), in which arsenic exposure was also associated with increased leukocyte DNA methylation.

The markers of DNA methylation were also different; Pilsner and colleagues[5] used the methyl acceptance assay to measure genomic DNA methylation in blood leukocytes, while we used pyrosequencing to measure Alu and LINE-1 repetitive element methylation. Although Alu and LINE-1 have both been considered surrogate measures of global methylation because they capture the global hypomethylation found in cancer tissues, DNA methylation in these elements could differ from methylation in the rest of the genome. It is clear that patterns of methylation differ between LINE-1 and Alu. For example, methylation of LINE-1, but not Alu, was previously associated with traffic particles in this cohort.[35] It is also uncertain whether the two measures reflect global DNA methylation in peripheral blood methylation. A recent study analyzing 37 blood DNA samples from a case-control study of breast cancer failed to show any correlation between LINE-1 methylation and global DNA methylation.[36] Lastly, our study population was exposed to very low arsenic levels through the environment, while the Bangladesh study participants were chronically exposed to heavily arsenic-contaminated drinking water.

To our knowledge, our study is the first to examine the association between arsenic and repetitive element DNA methylation. Alu and LINE-1 elements, as retrotransposons, originate from RNA viruses and first transcribe to RNA, producing a ribonucleoprotein complex with reverse transcriptase and endonuclease properties. This facilitates the insertion of the DNA copy back into the genome at a new location, potentially even on a different chromosome. Retrotransposition may lead to constitutive safeguards to maintain the genome integrity by reducing or regulating the expression of these elements, which may be why more than one-third of DNA methylation occurs in these sequences.[37] However, recent evidence has shown they can be transcribed and translated into functional proteins,[38] and it is possible that the environment affects methylation in these sequences, resulting in their activation or altered gene expression.

While a trend of an inverse relation was observed between arsenic and LINE-1 methylation, Alu methylation tended to increase with increasing arsenic, which contradicts our a priori hypothesis that arsenic is associated with decreased DNA methylation. However, blood DNA methylation in Alu elements was inversely correlated with LINE-1 DNA methylation, which might indicate that Alu DNA methylation patterns differ from the patterns in other substrates. Alu and LINE-1 elements are controlled by different mechanisms that are not fully understood.[16] Metals can generate cellular stress through ROS production, and cellular stressors have been shown to trigger different transcription patterns among Alu and LINE-1 elements.[16] It is possible that arsenic could affect Alu and LINE-1 elements in distinct, yet unknown, ways. It may be relevant that Alu and LINE-1 methylation have previously been reported to have opposite associations with the risk of incident cardiovascular disease, one of the potential effects of arsenic exposure.[3] Alu was positively associated with cardiovascular disease,[39] whereas LINE-1 methylation was negatively associated.[40] Arsenic exposure has been shown to influence the activity of DNA methyltransferases,[25,41] which could result in DNA hypo- or hypermethylation of repetitive elements. If hypermethylation occurs, then the proportion of methylated cytosines will increase. As Pilsner et al.[5] discuss, this could lead to higher mutation risk, because deamination of cytosines is more frequent in the presence of attached methyl-groups, a process that yields $C \rightarrow T$ transitions.[5] This pathway could potentially explain the carcinogenicity of arsenic.

We found an interaction between toenail arsenic and plasma folate. Alu DNA methylation increased in men with plasma folate below the median, while those with higher folate values had the same magnitude of association but in the opposite direction. This finding was contrary to our expectation that methylation would decrease with increasing arsenic in men with lower plasma folate concentrations. In order to be excreted, inorganic arsenic transforms to the organic forms of monomethylarsonic acid or dimethylarsinic acid through methylation reactions. These reactions require methyl-groups provided by SAM, through one-carbon metabolism. Folate and other B-complex vitamins such as $B_{12}$ and $B_6$, provide the coenzymes that participate in one-carbon metabolism, which produces SAM, the universal methyl-group donor for essential substrates such as DNA. Thus, the availability of methyl nutrients can confer susceptibility to the effects of arsenic through altered DNA methylation. Folate deficiency has been associated with both arsenic-induced skin lesions and decreased arsenic methylation,[42,43] but the mechanism through which arsenic may increase Alu DNA methylation in people with lower folate values is unknown.

The lack of stronger associations in our study may be attributed to various factors. Firstly, the low arsenic exposure levels in our study population may have limited our ability to see a stronger association. Our participants had toenail arsenic concentrations that ranged between 0.02 μg/g and 1.45 μg/g, similar to those of other U.S.-based study populations.[44,45] Occupational sources of arsenic were unlikely in this population, as most of them were retired at the time of the study. A primary route of human arsenic exposure is water,[46] and the major supply source of water among our study participants was the Massachusetts Water Resources Authority, in which arsenic is consistently undetectable (< 1.0 μg/L). Thus, arsenic exposure in our study population likely comes from other common but low-level environmental sources, such as food, air, soil and dust.[4] Arsenic can be detected in food such as fish and seafood,[47] and thus we explored whether the associations were potentially confounded by fish/seafood intake, but no confounding was detected (data not shown). Secondly, arsenic may influence DNA methylation only when methyl nutrients are reduced. This would be consistent with our observation of a tendency of greater associations between arsenic and Alu or LINE-1 DNA methylation among people with lower values of plasma folate, $B_{12}$ and $B_6$.

Previous epidemiologic studies examining whether arsenic affects global or gene-specific DNA methylation have used non-biomarker exposure measures (e.g. arsenic in water),[26] or biomarkers of recent exposure (blood or urine arsenic).[5] Toenails are considered a good biomarker of exposure in epidemiologic investigations because they are less susceptible to external contamination than other matrices such as hair[44]; they are keratin-rich tissue, and inorganic arsenic tends to accumulate in them due to its high affinity for sulfhydryl groups; toenail arsenic concentration reflects an integrated measure of arsenic incorporated in the human body from multiple exposure routes, including water, food, air, soil and dust;[48] the concentration is not influenced by individual differences in arsenic metabolism;[49] and the concentration is indicative of arsenic exposure from the preceding 12-18 months reflecting longer-term exposure than blood or urine measurements.[45] It is possible that longer-term arsenic exposure rather than recent exposure is more relevant to the adverse health effects of arsenic, including cancer, ischemic heart disease and neurologic deficits. In our cohort, toenail arsenic has been positively related to cardiovascular effects, such as systolic and diastolic blood pressure as well as pulse pressure and QT prolongation, which is a risk factor for arrhythmia and sudden cardiac death.[50,51] If these effects are mediated through DNA methylation changes, then a measure of longer-term arsenic exposure is more appropriate when examining the association between arsenic and DNA methylation, which has been found to remain rather stable within individuals.[16]

Our study has several limitations. We did not measure DNA methylation status in arsenic's target organ tissues but rather in blood leukocytes, which may not represent methylation in target tissues. However, studies among subjects exposed to arsenic-contaminated water[26] and patients with arsenic-induced skin lesions[42] have found changes in gene-specific and genomic DNA methylation from peripheral blood leukocytes. Also, arsenic is an effective therapy for acute promyelocytic leukemia, indicating that arsenic distributes to these cells and influences their cellular function.[52] Furthermore, normal DNA methylation patterns have not yet been established,[30] but in order to determine them, it is essential to use easily accessible tissues such as blood.

A major strength of our study is that we explored the association of interest in a group of men environmentally exposed to arsenic levels that are representative of other US populations. Thus, our findings can be generalized to adult men exposed to arsenic through everyday life activities. We used an accurate quantitative analysis with pyrosequencing technology for measuring DNA methylation at more than one CpG dinucleotide loci. Additionally, our biomarker of arsenic exposure has been well validated as an integrated measure of exposure from multiple sources,[48] and it reflects medium to long-term exposure, which is probably more appropriate than recent exposure, given the known adverse health effects of arsenic. Furthermore, obtaining toenail samples is a non-invasive and convenient procedure for epidemiologic studies.

In conclusion, we observed a trend of increasing Alu and decreasing LINE-1 DNA methylation with increasing arsenic in a population of men with generally low arsenic exposure. Plasma folate seems to act as an effect modifier of the association between arsenic and Alu DNA methylation. While the mechanisms remain unclear, this finding indicates that the availability of methyl-nutrients could play a significant role in arsenic toxicity, if the latter is indeed mediated by DNA methylation. Future studies are needed to identify normal DNA methylation patterns in repetitive elements and to examine any relation of environmental exposures with deviations from the normal DNA methylation status. Identifying the various mechanisms that control Alu and LINE-1 elements will also shed light on how environmental stimuli affect repetitive sequences. In vitro and animal studies can provide valuable information on whether arsenic-induced epigenetic modifications in blood leukocytes are associated with changes in target tissues. Finally, determining whether epigenetic changes mediate the effect of arsenic on human health and whether this effect is less profound when methyl-nutrient levels are sufficient will suggest strategies for decreasing arsenic toxicity.

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## eAppendix 1

To remove extraneous contaminants, each sample was pre-cleaned before analysis by the following procedure: the samples were sonicated for 15 minutes in approximately 10 mL of 1% Triton X-100 solution in 15-mL plastic tubes, rinsed several times with distilled

deionized water, dried at 60°C for 24 hours in a drying oven and weighed into a 15-ml plastic tube. The samples were then digested with 1 ml of concentrated $HNO_3$ acid for 24 hours, diluted to 5-ml with deionized water and analyzed by ICP-MS (Elan 6100, Perkin Elmer, Norwalk, CT) using an external calibration method with tellurium as the internal standard for As.

Quality control (QC) measures included analyzing the initial calibration verification standard [standard reference material 1643e (trace elements in water); National Institute of Standards and Technology Gaithersburg, MD], continuous calibration standards, a solution of 1 ng/ml mixed-element standard As solution (NIST traceable), and a procedural blank. The QC sample was Certified Reference Material GBW 07601. Recovery of the QC standard analysis was 90% -110% with 95% precision.

The inter-assay coefficient of variation was 0.1. The detection limit for the analytical solution was 0.2 ng/mL. Due to sample weight variability (range: 0.002g-0.9g), the samples DL varied from 0.001 μg/g to 0.42 μg/g (mean = 0.02 μg/g) (sample DL= DL for the analytical solution x dilution factor).

## eAppendix 2

The structure of the fitted models is described by the following equation:

$$Y_{ijk}=\beta_0+u_k+\beta_E{}^{*}\text{Toenail As}_{jk}+\beta_L{}^{*}\text{CpG locus}_{ijk}+\beta' X_{jk}{}'+e_{ijk}$$

where $Y_{ijk}$ is the DNA methylation level in either Alu or LINE-1 repetitive element CpG locus i at time j in subject k; $\beta_0$ is the overall intercept; $u_k$ is the random intercept for subject k; $\beta_E$ is the slope representing the fixed effect of toenail As; $\beta_L$ is the slope representing the fixed effect of the CpG dinucleotide locus; β' is the vector of the regression coefficients for the covariates (vector of $X_{jk}$) at time j for subject k adjusted in the model; and $e_{ijk}$ is the residual error term.

## eAppendix 3

The influence diagnostics included measures of overall influence (i.e. likelihood distance), measures of influence on the estimates of the fixed effects and of the covariance parameters (i.e. Cook's D and the multivariate DFFITS statistic), measures of influence on the precision in the estimates of the fixed effects and of the covariance parameters (i.e. covariance trace and covariance ratio), measures of changes in the effect estimate of interest when removing participants or observations.

## eAppendix 4

Wright et al. found a negative association between patella lead and LINE-1 DNA methylation but not Alu.[1] Unfortunately, there was a great amount of missing information on patella lead measurements in our specific study population with only 181 study visits (from a total of 735) having available patella lead measurements. To evaluate potential confounding by lead, we examined the correlation between toenail arsenic and patella lead. We found that there was no correlation (spearman correlation coefficient ($r_s$)= -0.07, p-value=0.350, n=181). The half life of bone lead is ≥10 years and thus bone lead is considered a measure of long-term exposure. Therefore, we believed that it would be reasonable to assign this long-term measure of exposure to the visits of the same individual that were relatively close in time in order to increase the number of visits with available

bone lead measurements. We assigned the immediate previous (if previous was not available, we assigned the immediate next in time) patella lead measurement to the missing values of patella lead. For those individuals who missed both, the missing value remained missing. After doing that, the number of visits with available patella lead measurements was increased to 429 (from a total of 735) and again no correlation between patella lead and toenail arsenic was detected [$r_s$= -0.04, p-value=0.618 at baseline visit (n=168) and $r_s$=-0.03, p-value=0.516, for all visits (n=429)]. Since lead exposure was not associated with As exposure, we did not further considered this covariate in our analysis.

A negative association was found between black carbon exposure and Alu DNA methylation as well as between $SO_4$ and LINE-1 DNA methylation by Madrigano et al. among NAS participants.[2] Specifically, an interquartile range increase in BC over a 45-, 60- and 90-day period was associated with a decrease of 0.17% (95%CI, -0.34 to 0.00), 0.21% (95%CI, -0.39 to -0.03) and 0.31% 5-methylcytosine (95% CI, 0.12-0.50%) in Alu respectively. An interquartile range increase in $SO_4$ over a 90-day period was associated with a decrease of 0.27% 5mC (0.02-0.52%) in LINE-1. In order to examine the association between the air pollutants black carbon and $SO_4$ and our exposure of interest, we calculated the spearman correlation coefficients between toenail arsenic with the 45-, 60-, and 90-day moving average of black carbon as well as the 90-day moving average of $SO_4$ and the results are presented in eTable 1. Given the lack of association between toenail arsenic and black carbon, we did not consider this covariate further in our analysis.

Since the 90-day moving average for $SO_4$ was associated with toenail arsenic, we included the covariate in our models for LINE-1 to check whether it will influence our findings. Since there were missing $SO_4$ measurements, we re-ran our models using only the visits which have data on $SO_4$ and the results for the main effect of toenail arsenic original model and the model additionally adjusted for $SO_4$ are presented in eTable 2. The main effect of toenail arsenic on LINE-1 DNA methylation in this restricted population did not change when $SO_4$ was included in the model.

Also, Baccarelli et al. have also reported a negative association between recent higher black carbon and PM2.5 exposure (7-d moving average) and LINE-1 methylation.[3] We explored whether there was an association between toenail arsenic and both 7-day averages of black carbon and PM2.5 in our study population and we found no correlation between arsenic and black carbon ($r_s$=-0.00, p-value=0.997, n=735) or arsenic and PM2.5 ($r_s$=-0.00, p-value=0.953, n=735). No correlations were also found at first visit only.

## Appendix

**eTable 1**

Spearman correlation coefficients between toenail arsenic and air pollutants among the study population

| Air Pollutant (average) | Number of visits | Spearman r | p-value |
|---|---|---|---|
| Black carbon | | | |
| 45 days | 735 | 0.05 | 0.202 |
| 60 days | 735 | 0.02 | 0.600 |
| 90 days | 735 | -0.02 | 0.673 |
| $SO_4$ | | | |
| 90 days | 594 | 0.10 | 0.014 |

# Appendix

**eTable 2**

Estimated changes in LINE-1 DNA methylation associated with one interquartile change (0.06μg/g) in toenail arsenic among the study population with complete data on $SO_4$ (n=1782 CpG methylation observations from 594 visits).

| LINE-1 | Observations | Beta (95% CI) |
|---|---|---|
| Original Model[a] | 1782 | 0.02 (-0.06 to 0.09) |
| Additionally adjusted for $SO_4$ | 1782 | 0.02 (-0.06 to 0.09) |

[a]Multivariable adjusted mixed effects model adjusted for age, laboratory batch, CpG locus number, % lymphocytes, alcohol drinking status, and body mass index.

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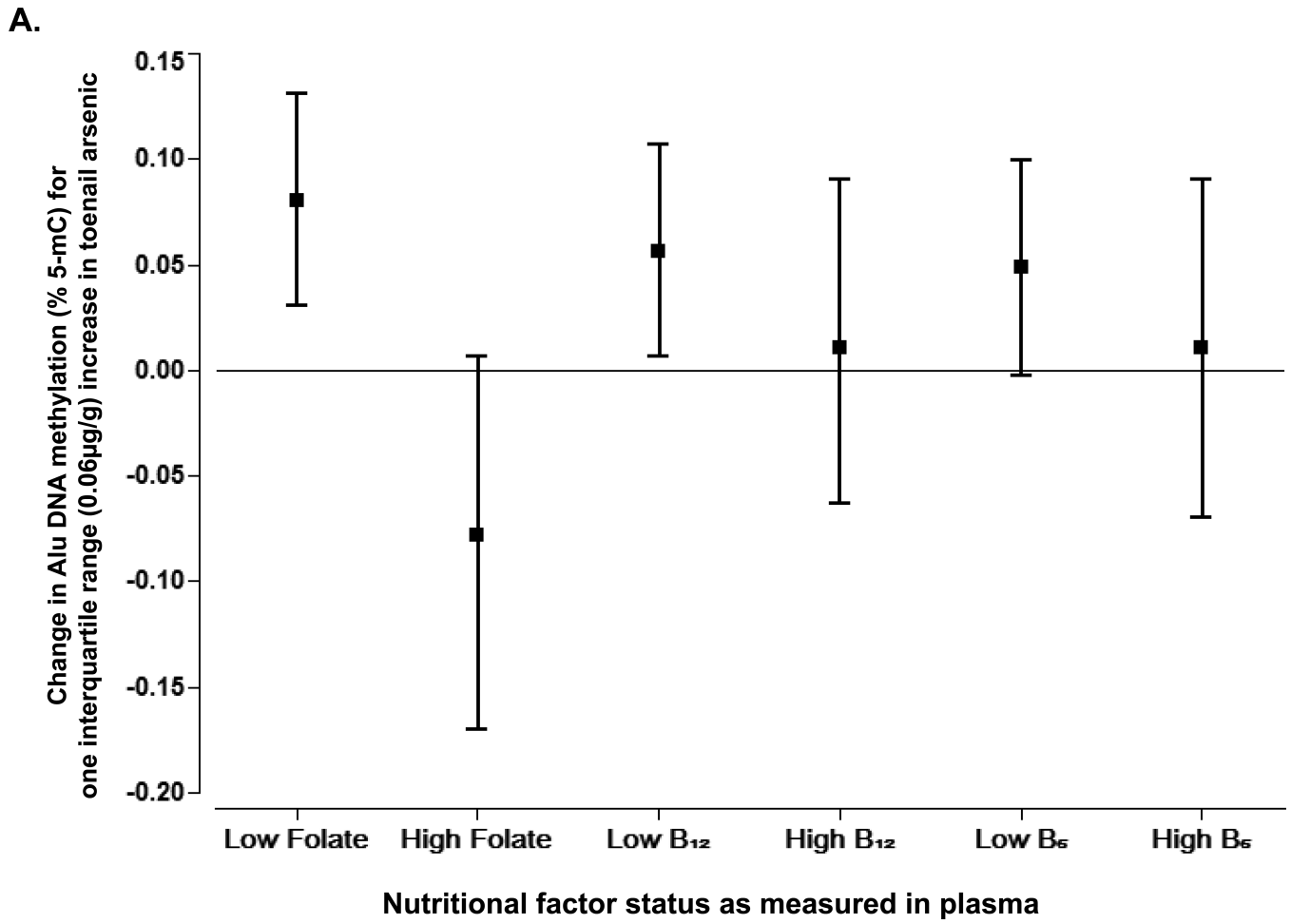

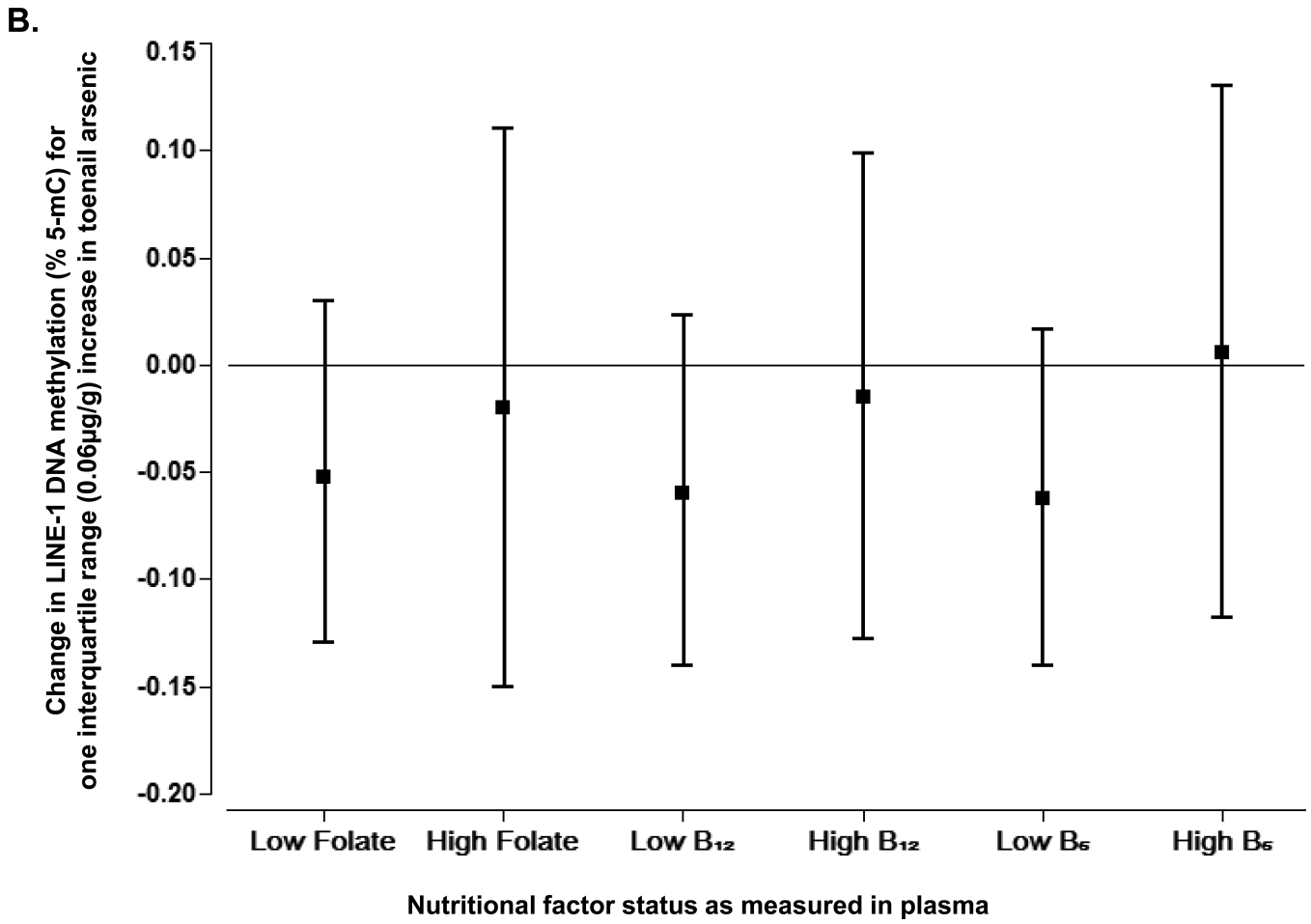


**FIGURE.**

Estimated change in (A.) Alu and (B.) LINE-1 DNA methylation (% 5-mC), with 95% confidence interval, per one interquartile increase (0.06µg/g) in toenail arsenic, by nutritional factor status as measured in plasma. The median values of plasma concentrations of folate, cobalamin (vitamin $B_{12}$) and pyridoxine (vitamin $B_6$) were used to divide participants in low and high groups. The median values of plasma folate, vitamin $B_{12}$ and vitamin $B_6$ were 14.1 ng/ml, 475.5 pg/ml, and 72.7 nmol/L, respectively. All models were adjusted for age, laboratory batch, CpG locus number, percent lymphocytes, alcohol-drinking status, and body mass index.

## TABLE 1

Characteristics[a] of the study population at baseline visit (n=581) and by tertiles of toenail arsenic, 1999-2002 & 2006-2007

| | | Tertiles of toenail arsenic (μg/gr) | | |
|---|---|---|---|---|
| **Characteristic** | **All (n=581)** | **≤0.061 (n=194)** | **0.062-0.096 (n=194)** | **>0.096 (n=193)** |
| Age (years) | 72.0 (9.0) | 73.0 (11.0) | 69.0 (9.0) | 71.0 (9.0) |
| Body mass index (kg/m$^2$) | 27.5 (4.7) | 27.7 (5.3) | 27.5 (5.0) | 27.3 (3.9) |
| Lymphocytes (%) | 25.0 (10) | 24.0 (11) | 25.0 (9) | 26.0 (9) |
| Plasma folate (ng/mL)[b] | 13.3 (10.9) | 15.3 (12.6) | 13.2 (10.4) | 12.4 (9.4) |
| Plasma vitamin $B_{12}$ (pg/mL)[b] | 475.0 (262.5) | 483.5 (278.0) | 492.5 (255.5) | 446.5 (231.0) |
| Plasma vitamin $B_6$ (nmol/L)[c] | 72.3 (80.6) | 71.0 (76.1) | 64.5 (65.5) | 82.8 (85.2) |
| Pack years | 10.0 (31.3) | 9.8 (30.0) | 11.1 (31.0) | 10.0 (30.0) |
| Smoking status; No. (%) | | | | |
| Never | 175 (30) | 66 (34) | 50 (26) | 59 (31) |
| Former | 386 (67) | 122 (63) | 138 (71) | 126 (65) |
| Current | 20 (3) | 6 (3) | 6 (3) | 8 (4) |
| Alcohol drinking; No. (%) | | | | |
| >= 2 drinks/day | 112 (19) | 18 (9) | 45 (23) | 49 (25) |
| < 2 drinks/ day | 469 (81) | 176 (91) | 149 (77) | 144 (75) |
| Season of visit; No. (%) | | | | |
| Spring | 160 (27) | 67 (35) | 49 (25) | 44 (23) |
| Summer | 160 (28) | 43 (22) | 53 (27) | 64 (33) |
| Fall | 172 (30) | 43 (22) | 59 (31) | 70 (36) |
| Winter | 89 (15) | 41 (21) | 33 (17) | 15 (8) |
| Alu methylation (% 5-mC)[d] | 26.0 (1.3) | 26.1 (1.4) | 26.0 (1.2) | 25.8 (1.2) |
| CpG position 1 | 35.0 (2.1) | 35.0 (2.2) | 35.1 (1.9) | 34.8 (2.2) |
| CpG position 2 | 25.8 (1.6) | 26.0 (1.6) | 25.7 (1.7) | 25.7 (1.5) |
| CpG position 3 | 16.8 (1.4) | 17.0 (1.5) | 16.8 (1.1) | 16.7 (1.5) |
| LINE-1 methylation (% 5-mC)[d] | 77.3 (2.8) | 77.1 (2.3) | 77.2 (2.8) | 77.6 (3.7) |
| CpG position 1 | 82.3 (3.0) | 82.2 (2.4) | 82.0 (3.0) | 82.8 (3.1) |
| CpG position 2 | 75.1 (3.4) | 74.8 (2.8) | 75.2 (3.6) | 75.4 (3.9) |
| CpG position 3 | 74.8 (3.5) | 74.3 (3.0) | 74.8 (3.6) | 75.0 (4.5) |

[a] Median (IQR), unless otherwise indicated

[b] Available for 544 subjects (182, 180 and 182 subjects in the 1st, 2nd, and 3rd tertile of arsenic distribution, respectively)

[c] Available for 539 subjects (181, 181 and 177 subjects in the 1st, 2nd, and 3rd tertile of arsenic distribution, respectively)

[d] The average of the three CpG loci DNA methylation

## TABLE 2

Estimated change in DNA methylation in Alu and LINE-1 repetitive elements associated with an interquartile range (0.06 μg/g) increase in toenail arsenic concentrations among Normative Aging Study participants, 1999-2002 & 2006-2007 (n=2205 observations).

| Outcome | Change in methylation for an IQR change in toenail Arsenic (μg/g) | (95% CI) |
|---|---|---|
| Alu DNA methylation (% 5-mC) | | |
| Model 1[a] | 0.03 | (-0.01 to 0.07) |
| Model 2[b] | 0.03 | (-0.01 to 0.07) |
| Model 3[c] | 0.03 | (-0.01 to 0.07) |
| LINE-1 DNA methylation (% 5-mC) | | |
| Model 1[a] | -0.05 | (-0.11 to 0.02) |
| Model 2[b] | -0.05 | (-0.11 to 0.02) |
| Model 3[c] | -0.05 | (-0.11 to 0.02) |

[a]Model 1 is a multivariable mixed effects models adjusted for age, laboratory batch, CpG locus number, percent lymphocytes, drinking status, and body mass index.

[b]Model 2 is model 1 additionally adjusted for smoking status and pack-years.

[c]Model 3 is model 2 additionally adjusted for season and day of the week.

## TABLE 3

Estimated changes in Alu and LINE-1 DNA methylation associated with changes in the covariates among Normative Aging Study participants with complete data on plasma folate and vitamins $B_{12}$ and $B_6$, 1999-2002 & 2006-2007 (n=2064 observations).

| Covariate | Alu (% 5-mC) β (95% CI)[a] | LINE-1 (% 5-mC) β (95% CI)[a] |
|---|---|---|
| Model 1 | | |
| Toenail Arsenic (μg/g) | -0.08 (-0.17 to 0.01)[c] | -0.02 (-0.15 to 0.11)[c] |
| Low folate[b] | -0.30 (-0.53 to -0.06) | 0.24 (-0.12 to 0.61) |
| Toenail Arsenic *Low folate | 0.16 (0.06 to 0.26) | -0.03 (-0.19 to 0.12) |
| Model 2 | | |
| Toenail Arsenic (μg/g) | 0.01 (-0.06 to 0.09)[c] | -0.26 (-2.21 to 1.70)[c] |
| Low vitamin $B_{12}$[b] | -0.22 (-0.44 to 0.01) | 0.22 (-0.13 to 0.56) |
| Toenail Arsenic *Low vitamin $B_{12}$ | 0.05 (-0.04 to 0.13) | -0.69 (-2.96 to 1.58) |
| Model 3 | | |
| Toenail Arsenic (μg/g) | 0.01 (-0.07 to 0.09)[c] | 0.01 (-0.12 to 0.13)[c] |
| Low vitamin $B_6$[b] | 0.08 (-0.15 to 0.31) | 0.34 (-0.02 to 0.70) |
| Toenail Arsenic *Low vitamin $B_6$ | 0.04 (-0.06 to 0.13) | -0.07 (-0.22 to 0.08) |

β indicates regression coefficient.

[a] From multivariable mixed effects models additionally adjusted for age, laboratory batch, CpG locus number, percent lymphocytes, alcohol drinking status, and body mass index.

[b] The median values of plasma concentrations of folate, cobalamin (vitamin $B_{12}$) and pyridoxine (vitamin $B_6$) were used to divide participants in low and high groups. The median values of plasma folate, vitamin $B_{12}$ and vitamin $B_6$ were 14.1ng/ml, 475.5pg/ml, and 72.7nmol/L, respectively.

[c] Estimated change in Alu or LINE-1 DNA methylation for an interquartile range (0.06μg/g) increase in toenail arsenic concentration.