Elevated lactate dehydrogenase activity and increased cardiovascular mortality in arsenic-endemic areas in southwestern Taiwan

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Abstract

Arsenic ingestion has been linked to increasing global prevalence of and mortality from cardiovascular disease (CVD); arsenic can be removed from drinking water to reducte related health effects. Lactate dehydrogenase (LDH) is used for the evaluation of acute arsenic toxicity *in vivo* and *in vitro*, but it is not validated for the evaluation of long-term, chronic arsenic exposure. The present study examined the long-term effect of chronic arsenic exposure on CVD and **serum** LDH levels, after consideration of genetic susceptibility and arsenic metabolism capacity. A total of 380 subjects from an arseniasis-endemic area and 303 from a non-endemic area of southwestern Taiwan were recruited in 2002. Eight functional polymorphisms in *PON1, PON2, AS3MT, GSTO1,* and *GSTO2* were assessed for genetic susceptibility in relation to the arsenic-related LDH elevation. Various urinary arsenic species were analyzed using high-performance liquid chromatography (HPLC) and hydride generation systems. Fasting plasma was used for quantitative determination of the total LDH activity. A significant dose-response relationship was observed between arsenic exposure and LDH elevation, independent of genetic polymorphisms and urinary arsenic profiles ($P < 0.001$). Furthermore, abnormal LDH elevation was associated with CVD mortality after adjustment for Framingham risk scores for 10-year CVD and arsenic exposure (hazard ratio, **6.56**; 95% confidence interval,

1.39–31.07). LDH was elevated in subjects with arsenic exposure in a dose-dependent manner. LDH is a marker of arsenic toxicity associated with CVD mortality. Results of this study have important implications for use in ascertaining long-term arsenic exposure risk of CVD.

Keywords

Arsenic exposure, Lactate dehydrogenase, Cardiovascular mortality, Paraoxonase, Arsenic methyltransferase, Glutathione S-transferases omega

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Introduction

Arsenic is a potent but modifiable environmental pollutant that has been linked to the increasing prevalence of cardiovascular disease (CVD), a major cause of excess mortality worldwide (Navas-Acien et al., 2005); arsenic can be removed from drinking water to reduce related health effects. Nonetheless, little was known about the excess mortality from arsenic when genetic factors were considered, notwithstanding individual susceptibility to arsenic toxicity (NRC, 1999) due to differences in age, sex, and arsenic metabolism (Vahter, 2000; Watanabe et al., 2001). Inter-individual differences in the speciation and amounts of arsenic metabolites are reported among subjects chronically exposed to arsenic (Loffredo et al., 2003) and significant genetic determinants of arsenic metabolism are supported by

epidemiologic evidence (Chung et al., 2002). Arsenic-induced CVD may result from the inter-correlations among genetic, environmental, and toxicological mechanisms. **The total level of plasma lactate dehydrogenase (LDH), which measures all five isoforms (LDH1 to LDH5) present in the blood, is usually used in the diagnosis and treatment of acute tissue damage, cardiac diseases such as acute myocardial infarction (Galen et al., 1975), tumors of the lung (Turan et al., 2007), and liver diseases such as acute viral hepatitis, cirrhosis, and metastatic carcinoma of the liver or kidneys (Chu et al., 2002). In addition, LDH has also been shown to predict in-hospital mortality among subjects with severe underlying physical illnesses *{when used in addition to assessment of functional status} (Davis *et al.*, 1995).** [Dear Author: *I do not understand why you have the phrase in brackets as it does not seem to have anything to do with the rest of the sentence. Is the revision of the phrase in brackets accurate according to your meaning?] Furthermore, LDH has also been widely used for the evaluation of acute arsenic toxicity *in vivo* and *in vitro* (Petrick et al., 2000; Peraza et al., 2003; Saad et al., 2006; Tajima et al., 2010); but the association between chronic arsenic exposure and the level of **serum** LDH remains unclear. One recent cross-sectional study showed that the total LDH level in the plasma correlated positively with the concentration of arsenic in drinking water (Karim et al., 2010). Therefore, in conjunction with the role of LDH in cardiovascular

evaluation, we suggest a link between arsenic-related LDH elevation and the mortality of CVD.

Not until recently have genes encoding enzymes responsible for arsenic metabolism been cloned and characterized (Whitbread et al., 2003; Wood et al., 2006). They include *AS3MT* and *GSTO*. The *AS3MT* gene directly encodes a cytosolic enzyme, arsenic methyltransferase, which catalyzes a multi-step process to convert inorganic arsenic to monomethyl arsenical (MMA) and dimethyl arsenical (DMA) (Lin et al., 2002). **The polymorphism M287T (rs17885947) in *AS3MT* is considered to be related to inter-individual variation in arsenic metabolism (Drobna *et al.*, 2004).** Glutathione S-transferases (GSTs) are phase II detoxification enzymes that catalyze the conjugation of reduced glutathione to a wide variety of endogenous and exogenous electrophilic compounds (Townsend and Tew, 2003). The GST omega class is a subfamily of GSTs shown to be identical with human monomethylarsonic acid (MMA) reductase, the rate-limiting enzyme for biotransformation of inorganic arsenic. Polymorphisms of the GST omega genes are associated with intracellular thiol status and the arsenic biotransformation efficiency in liver cells (Tanaka-Kagawa et al., 2003). **A140D (rs4925) in *GSTO1* and N142D (rs156697) in *GSTO2* have been identified as common polymorphisms in different ethnic populations (Mukherjee *et al.*, 2006; Polimanti *et al.*, 2011). A140D has also been shown to**

reduce enzyme activity and *inhibit inorganic arsenic biotransforming capacity (Tanaka-Kagawa et al., 2003). [Dear Author: *Is this what you mean?]

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High-density lipoprotein (HDL) is postulated to prevent the development of atherosclerosis by inhibiting the oxidation of low-density lipoprotein (LDL). Human paraoxonase (PON1) is a serum esterase/lactonase transported on HDL particles and the major determinant of the antioxidant action of HDL (Aviram et al., 1998). Both in *in vitro* and animal studies using *PON1*-knockout mice showed that PON1 prevented LDL oxidation and is, therefore, a protective enzyme against the development of atherosclerosis (Mackness et al., 1991; Watson et al., 1995; Shih et al., 1998; Li et al., 2009). **The *PON1* gene has two common polymorphisms, L55M (rs854560) and Q192R (rs662), within the coding region resulting in amino acid substitutions, and one common polymorphism, C-108T (rs705379), in the promoter region, *{which have all } have been associated with PON1 levels (Adkins *et al.*, 1993; Leviev and James, 2000; Furlong *et al.*, 2005).** [Dear Author: *Is the phrase in brackets accurate?] **A148G (rs12026) and C311S (rs6954345) in *PON2* have also been identified and are considered to be associated with lipid profiles including levels of total cholesterol, low-density lipoprotein, and apolipoprotein A1 and B (Boright *et al.*, 1998; Hegele, 1999). Animal study has demonstrated that atherosclerosis is induced by arsenic in drinking water through alteration of lipid**

metabolism (Cheng *et al.*, 2011). Additionally, our previous studies showed significant genetic variation in the *PON* gene cluster, as well as in PON1 activity, electrocardiographic abnormalities, and increased intima medium thickness of the carotid artery in subjects who had long-term arsenic exposure (Li et al., 2009; Liao et al., 2009). The present study examined the long-term effect of chronic arsenic exposure and serum LDH levels on CVD after consideration of individual genetic susceptibility ???*{to arsenic and CVD, and urinary arsenic metabolism.} [Dear Author: The previous sentence is not clear and I could not guess what you meant, *especially the words in brackets. What is "urinary arsenic metabolism"? Do you mean "urinary arsenic metabolites"?] Our results are beneficial to the identification of a biomarker for long-term arsenic exposure risk assessment.

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Materials and methods

Study areas

The study included a community-based cohort comparing the population from a previously arseniasis-endemic area in southwestern Taiwan to a non-exposed population recruited from a documented non-endemic area in the same county. The subjects shared similar ages, genders, and ecological statuses at the time of data collection. The arseniasis-endemic area of Homei, Fusin, and Hsinming villages in

Putai Township on the southwestern coast of Taiwan were described previously (Chen et al., 1985; Chen et al., 1995; Tseng et al., 2003). In short, residents in the study area consumed contaminated artesian well water for decades since the 1910s, and the arsenic concentration in the water as measured in the early 60s ranged from 0.035 to 1.14 ppm, with a median of 0.78 ppm (Chen et al., 1962; Kuo, 1964). The total daily intake of arsenic by the locals was estimated as high as 1 mg (Blackwell, 1961), until a municipal water supply system was installed and commonly functional in the early 1970s. The arsenic concentration of tap water supplied in the study area was then reduced to less than 0.01 ppm (Chen and Chen, 1975). The non-endemic area was Chiali Township where the arsenic concentration of well water was under the detection limits according to surveys conducted in 1960s and 1970s (Kuo, 1964; Lo et al., 1977). The climate, ethnic background (Han Chinese), degree of urbanization and socioeconomic status were similar between Putai and Chiali.

Study subjects

In January 2002, approximately 490 subjects who still resided in the study area were invited to participate in this study. Frequency matching of the age and gender distributions (from 35- to 85- years old, in 5-year age groups) was conducted to recruit residents into the previously unexposed group. A total of 380

and 303 residents were enrolled for the present study respectively. Among these subjects, 380 and 296 from the endemic and non-endemic areas, respectively, who had complete biochemical measurements were included in the final analysis of mean baseline characteristics (Table 1) and baseline characteristics stratified by LDH levels (Table 2). Urinary arsenic species (Table 3) and DNA (Table 4) were analyzed in 343 and 291 subjects with these data from endemic and non-endemic areas, respectively. Multivariable association of abnormal LDH elevation was conducted in 316 and 275 subjects from endemic and non-endemic areas who had complete data (Table 5). Only subjects from endemic areas were included in the final analysis of association of abnormal LDH elevation with causes of death (Table 6).

Data collection

Physical measurements, including blood pressure, electrocardiograms and carotid artery imaging were collected. In addition, standardized personal interviews were conducted by public health nurses using a structured questionnaire to acquire baseline and socioeconomic characteristics, artesian well water usage, residential history, lifestyle variables, and personal and family histories of hypertension, diabetes, and CVD. Cumulative arsenic exposure (in

ppm-years) was calculated from the arsenic concentration in artesian well water (ppm) and the duration of water consumption (years). The cause of death for a deceased subject, as classified according to the 9th revision of International Classification of Diseases (ICD-9), was retrieved from the database of the National Death Certification System referenced to the subject's national identification number, with the approval from the Department of Health in Taiwan. All deaths that occurred during the time of study (from January 1, 2002 to December 31, 2009) were counted. The study protocol was approved by the Human Ethical Committee of the National Health Research Institutes in Taiwan and informed consent was obtained from each participant before starting the study.

Biochemical analysis

Fasting venous blood samples were collected for quantifying the **serum** total LDH levels using a Beckman SYNCHRON LX20 System (Beckman Coulter, Fullerton, CA, USA) according to the manufacture's protocol. The detection limit for the LDH assay was 5 IU/L. **Plasma glucose, serum levels of glycated hemoglobin A1c (HbA1c), lipid profiles (high density lipoprotein, low density lipoprotein, cholesterol, and triglycerides), liver function enzymes (aspartate aminotransferase and alanine aminotransferase), *microalbumin, uric acid and creatinine were also analyzed**

when the subjects participated in a health examination in 2002. [Dear Author: Do you mean “albumin” here? “Microalbumin” is usually measured in the urine.]

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Urinary samples were collected from each subject for arsenic species analyses. Subjects were asked not to consume seafood three days before urine collection. Arsenite (As(III)), arsenate (As(V)), MMA, and DMA were quantified using high-performance liquid chromatography (HPLC) coupled with flow injection atomic absorption spectrometry. The HPLC system consisted of a solvent delivery pump (PU-1580, Jasco, Tokyo, Japan) and a silica-based anion-exchange column (Nucleosil 10 SB, 250 mm×4.6 mm; Phenomenex, CA, USA) with a guard column packed with the same material. A flow injection analysis system (FIAS-400, PerkinElmer, CT, USA) was designed as the on-line interface to the continuous hydride generation system (Analyst 100, PerkinElmer) used in this study. With this method, the within- and between-day precision (coefficient of variation, CV%) for As(III), As(V), MMA, and DMA range from 1.0% to 3.7%. Furthermore, the recoveries for As(III), As(V), MMA, and DMA were 99.0, 98.9, 99.0, and 99.0%, while the detection limits were 0.75, 1.47, 1.19, and 0.76 μg/L, respectively. The primary methylation index was defined as the ratio between MMA and iAs (As(III) + As(V)) levels, and the secondary methylation index was defined as the ratio between DMA and MMA.

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SNP selection and genotyping

DNA was collected in 2002 and analyzed in 2005 in both the endemic and non-endemic areas . Eight functional polymorphisms for genotyping analysis were selected from the National Center for Biotechnology Information single nucleotide polymorphism (SNP) database based on their implications in arsenic metabolism, cardiovascular disease and minor allele frequency. These SNPs included C-108T (rs705379), L55M (rs854560) and Q192R (rs662) of *PON1*; A148G (rs12026) and C311S (rs6954345/rs7493) of *PON2*; M287T (rs17885947) of *AS3MT*; A140D (rs4925) of *GSTO1*; and N142D (rs156697) of *GSTO2*.

Genomic DNA was extracted from buffy coat using commercial kit (PUREGENE®, Gentra, Minneapolis, MN, USA). The AS3MT M287T polymorphism was determined using a commercially designed TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA). All other genotypes were determined by polymerase chain reaction amplification followed by polymorphism-specific restriction enzyme digestion analysis (RFLP-PCR). **For the A140D in *GSTO1*, N142D in *GSTO2*, and M287T in *AS3MT*, all specimens were repeated by RFLP-PCR. The kappa statistics were about 0.88 and the samples with discordant results were sent for DNA sequencing for genotype validation. For other SNPs, 20% of the samples were run in duplicate and all of the kappa statistics were > 0.94. All the samples**

were relabeled for the experiments and the researchers were blinded to individual identities and results.

Statistical analysis

Differences between the study subjects in endemic (Putai) and non-endemic (Chiali) areas were assessed for the association between demographic characteristics and cardiovascular risk factors. Continuous variables were expressed as means with standard deviations and evaluated using Student's *t*-test or the Wilcoxon rank-sum test. Categorical variables were expressed as proportions and compared using the chi-square test or Fisher's exact test. Histograms were used to present LDH distributions for the endemic and non-endemic areas. Since no clinical threshold is currently set for abnormal serum LDH level, we defined an abnormal LDH level as an elevation of two standard deviations above the mean LDH level of the subjects in the non-endemic area. Characteristics among subjects including urinary arsenic species were compared between groups with normal and abnormal LDH levels stratified by arsenic-endemic and non-endemic populations. Univariate analyses of LDH elevation in relation to genetic polymorphisms were performed based on logistic regression. A multiple regression model was then utilized to evaluate the independent association between arsenic exposure and abnormal LDH elevation.

Arsenic exposure in the endemic area was stratified into two categories by the median level in reference to the subjects in non-endemic area. **All-cause and CVD-caused deaths including cardiovascular disease (ICD-9: 390-429) and cerebrovascular disease (ICD-9: 430-438) were identified as mortalities of interest.** Multivariate Cox regression analysis was used to determine the hazard ratios for abnormal elevation of LDH levels and mortality of interest after adjustment for conventional risk factors, including age, gender, cigarette smoking, hypertension, and diabetes mellitus (D'Agostino *et al.*, 2008), using the Framingham risk score for 10-year cardiovascular disease as a covariate. *P*-values less than 0.05 were considered statistically significant. All statistical analyses were conducted using SAS 9.2 (SAS, Inc., Cary, NC).

Results

Descriptive statistics of study participants

The average characteristics of the study populations are summarized in Table 1. The numbers of study subjects in endemic area and non-endemic area were 380 and 296, respectively. Demographic data showed that there was no significant difference in the age and gender profiles between the two areas. However, the percentage of subjects who smoked cigarettes was significantly higher in the endemic area. **The average**

LDH levels for the non-endemic and endemic areas were 295.06 (IU/L) and 408.43 (IU/L), respectively. Additionally, the subjects in the endemic area had higher aspartate aminotransferase, alanine aminotransferase, LDL levels, and body mass index; their diastolic blood pressure, HDL, and uric acid levels were significantly lower, when compared to the subjects from the non-endemic area.

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[Table 1 here]

The Figure shows the histograms of the LDH levels among the study subjects. A great majority of the subjects (n=296; 77.8%) from the endemic area had LDH levels greater than 300 (IU/L). On the other hand, only 38.6% of subjects (n=117) from the non-endemic area had elevated LDH levels (>300 IU/L), while most of the subjects had LDH levels that ranged from 200 to 300 IU/L.

[Figure here]

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Univariate and multivariate associations with LDH elevation

The association of characteristics of study subjects and abnormal LDH elevation stratified by endemic and non-endemic areas is shown in Table 2. As mentioned

above, the critical threshold for abnormal LDH elevation in this study was calculated as the mean LDH level of subjects in the non-endemic plus two standard deviations, or 450 (IU/L). Based on this criterion, 12 out of 296 subjects from the non-endemic area and 97 out of 380 subjects from the endemic area were identified as having abnormal LDH elevation. Subjects with abnormal LDH elevation were significantally older than those with without LDH elevation in both the endemic and non-endemic areas. In addition, significantly elevated systolic blood pressure (SBP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and body mass index (BMI) and *{significanlty more microalbuminuria} were also seen in subjects with abnormal LDH levels in the endemic area but not in non-endemic area. [Dear Author: *Is this phrase in brackets correct? Is the previous sentence edited correctly?]

[Table 2 here]

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Table 3 shows a comparison of urinary arsenic species between subjects with normal and abnormal LDH levels stratified by arsenic-endemic and non-endemic areas. In subjects from the endemic area, urinary arsenic species of As(III), iAs, the sum of iAs and MMA, and the sum of iAs, MMA, and DMA were significantly higher in subjects with abnormal LDH elevation compared

with that of subjects with normal LDH levels.

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[Table 3 here]

Eight functional polymorphisms, i.e., C-108T, L55M and Q192R in *PON1*, A148G and C311S in *PON2*, M287T in *AS3MT*, A140D in *GSTO1*, and N142D in *GSTO2*, were screened for their association with abnormal LDH elevation. The Hardy-Weinberg equilibrium was calculated for the subjects with normal LDH levels in both endemic and non-endemic areas. The A140D and C-108T polymorphisms showed a significant departure from equilibrium; M287T and L55M had a minor allele frequency less than 5% and were thus removed from further analysis. Genotypic frequencies of C311S showed a significant association with abnormal LDH elevation in the endemic area, but not in the non-endemic area. Compared with the SS genotype, the CC genotype of C311S had a 5.91-time increased risk (95% CI: 1.70–20.55, p=0.005) of abnormal LDH elevation in the endemic area (Table 4).

[Table 4 here]

Multivariable analyses of the association between chronic arsenic exposure and abnormal LDH elevation are shown in Table 5. Arsenic exposure was evaluated by duration of arsenic water consumption (Model 1) and cumulative arsenic

(Model 2). Compared t with subjects from the non-endemic area, subjects in the endemic area with short durations (less than 21 years) and long durations (more than 21 years) of arsenic exposure had significantly increased odds ratios of abnormal LDH elevation, OR= 8.65 (95% CI: 3.68–20.34, p=0.002) and 9.11 (95% CI: 3.83–21.66, p=0.001), respectively. Similar results were also observed when cumulative arsenic exposure was considered. The odds ratios for abnormal LDH elevation for short (<14.7 ppm-years) and long cumulative exposures (>14.7 ppm-years) were 8.16 (95% CI: 3.57–18.64, p=0.004), and 9.59 (95% CI: 3.75–24.53, p=0.002), respectively. Moreover, variables including age, AST, and urinary levels of iAs and MMA were also associated with increased odds ratios of abnormal LDH elevation independent of other covariates in both models. In addition, gender, cigarette smoking, SBP, HDL, BMI and CC genotype of C311S polymorphism in *PON2* showed increased risks for abnormal LDH elevation but were not statistically significant.

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[Table 5 here]

Association between abnormal LDH elevation and mortality

During the 7-year follow-up, 45 subjects died from all-cause mortality including 43 subjects from physical illness- related deaths: 17 from neoplasms (ICD-9:

140–239), 10 from CVD, including cardiovascular disease (ICD-9: 390–429) and cerebrovascular disease (ICD-9: 430–438), 6 from respiratory disease (ICD-9: 460–519), 5 from endocrine disease (ICD-9: 240–279), and 4 from other systemic diseases including 1 from infectious disease, 1 from genitourinary disease, and 2 from external causes. One subject died from an accident and one from a homicide.[Dear Author: Is the previous sentence accurate according to your meaning?]Eight subjects from the non-endemic area died from physical illness-related deaths including 4 from neoplasms, 2 from CVD, 1 from respiratory disease and 1 from infection. Because of the diversity and limited number of deaths, the non-endemic area was not included in the final analysis of mortality.

[Dear Author: Is the previous sentence accurate according to your meaning?] **Only the associations of abnormal LDH elevation with all-cause mortality, excluding the accidental death and homicide, which are not conventional natural causes, and CVD-caused mortality in subjects in the arsenic-endemic area are summarized in Table 6. After adjustment for cumulative arsenic exposure and conventional risk factors for CVD by the Framingham risk score for 10-year CVD, abnormal LDH elevation was associated with increased risks of all-cause mortality (HR=3.07, 95% CI: 1.41–6.69) and CVD mortality (HR=6.56, 95% CI: 1.39–31.07). Furthermore, a longer cumulative exposure to arsenic (>14.7**

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ppm-years) was also associated with increased risk of all-cause (HR=1.09, 95% CI: 1.06–1.13) and CVD mortality (HR=1.07, 95% CI: 0.99-1.14). In addition, patients with the CC genotype of C311S polymorphism in *PON2* showed an increased risk for all-cause mortality with marginal statistical significance (HR=3.77, 95% CI: 0.78-18.24), but this was not analyzed in CVD mortality because of the small sample size (all subjects with CVD mortality carried SS genotypes).

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[Table 6 here]

Discussion

The present study examined the association between chronic arsenic exposure and LDH elevation. Previously, Brancaccio et al. (Brancaccio *et al.*, 2008) reported that factors associated with LDH levels included age, gender, race, muscle mass, physical activity, and climatic conditions. Our study further demonstrated that urinary arsenic species were associated with LDH elevation, especially As(III) and MMA, after adjustment for the reported conventional risk factors. We also examined the sum of iAs and MMA, both of which are indicators of inorganic arsenic exposure (Navas-Acien et al., 2008; Longnecker, 2009). **Multiple logistic regression analysis showed a link between urinary arsenic consisting of iAs and MMA and abnormal**

LDH elevation, independent of chronic arsenic exposure as well as underlying health status including the SBP, AST, HDL and BMI.

Additionally, we observed an increased risk of elevated LDH from *PON2* C311S genotypes in the arsenic-exposed subjects, suggesting that *PON2* genotype could affect arsenic toxicity as reflected in LDH activity. **In addition, subjects with missing genotyping were evaluated and showed no significant association with abnormal LDH levels, assuring the independent extent of missing data on genotyping.** [Dear Author: Is the previous sentence accurate according to your meaning? It is not very clear.] **The 311C allele in *PON2* is associated with increased risks of coronary artery disease, myocardial infarction, and also diabetic nephropathy (Pinizzotto et al., 2001; Martinelli et al., 2004; Jalilian et al., 2008); it is also reported to be associated with lower PON activity (Stoltz *et al.*, 2009). Recent studies have demonstrated that low PON1 activity is associated with increased risks of arsenic-induced atherosclerosis (Li et al., 2009) and is a successful predictor of cardiovascular diseases such as myocardial infarction and stroke (Mackness *et al.*, 2003; Bhattacharyya *et al.*, 2008).** Our data indicate the significance of the *PON2* C311S polymorphism during the pathogenesis of CVD, especially after chronic arsenic exposure, and therefore, suggest that cardiovascular damage should be monitored in subjects with arsenic poisoning.

Furthermore, we found that the risk of LDH elevation occurred in a dose-response relationship. The highest risk for abnormal LDH elevation was found among subjects with markedly long arsenic exposure, while a low risk was found among those never exposed. The elevation of LDH in the study areas also showed a statistically significant association with CVD mortality. The same was found after adjustment by the Framingham risk score for 10-year CVD risk factors, including age, gender, cigarette smoking, hypertension, and diabetes. While this finding could support for an underlying causal relationship between CVD and arsenic poisoning irrespective of other conventional risk factors, it is essential to note that LDH should be routinely monitored for clinical examinations, particularly in arsenic endemic areas in many parts of the world. **Previous epidemiologic studies have demonstrated liver toxicity and *{elevated liver function enzymes in the serum} in a dose-response relationship with arsenic exposure (Mazumder, 2005; Islam et al., 2011).** [Dear Author: *If you mean "AST and ALT" here, please use these enzyme names instead of this phrase in brackets.] **In the current study, AST and ALT levels were significantly higher among arsenic exposed subjects compared with those from the non-endemic area. AST levels were also associated with abnormal LDH elevation, especially among subjects in the arsenic-endemic area. These findings might be helpful for the early detection of arsenic-related liver disease.**

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We note several limitations, which should be considered when interpreting the results.

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First, the data were collected decades after the cessation of arsenic exposure. **Individual assessment of arsenic exposure at the time when exposure was *on-going was not conducted.** [Dear Author: *Is this what you mean?] **Instead, the exposure assessment in this study was performed in an ecological manner. Prior epidemiologic studies have demonstrated a positive correlation of cumulative arsenic exposure and the body burden of inorganic arsenic in Taiwan, even after residents had not consumed arsenic contaminated water for decades (Hsueh *et al.*, 1998; Huang *et al.*, 2007). Urinary arsenic data were presented as an indicator for accumulation of body burden as individual exposure data, however, the specific mechanism awaits further investigation.**

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Second, LDH is a general marker for cell toxicity and organ damage. **An elevation of the LDH level may indicate the overall health condition caused by arsenic toxicity, but may not be specific enough to pinpoint the target tissue(s). This study investigated the association between arsenic-related LDH elevation and all-cause mortality coded as physical illness-related deaths, an inherently heterogeneous category.** The total LDH level could only be used as a preclinical indicator in this study because the LDH **isoform** subtype was not specified. Clinical heterogeneity could result in dismissing this finding when different pathological

conditions are considered. **Recently, other cardiac markers such as creatine kinase MB (CK-MB), B-type natriuretic peptide (BNP) and troponins have been favored over LDH because of its low specificity.[Dear Author: Is the previous sentence accurate according to your meaning?]**

Nonetheless, if we restricted the mortality to cardiovascular disease only, the findings remained similar and the adjusted hazard ratio reached 6.56 (95% CI: 1.39–31.07). One particular study of 11,746 subjects in Bangladesh with current exposure to arsenic-contaminated drinking water showed a significantly increased risk of CVD mortality during an average of 6.6 years of follow-up (Chen *et al.*, 2011). In addition, a synergistic effect between cigarette smoking and arsenic exposure was identified in CVD mortality, especially from heart disease. However, we could not evalutate this synergistic association in the current study because of the limited sample size.

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Third, the Framingham risk score was applied to handle multiple comparison issues when a number of conventional risk factors were to be considered simultaneously. Although the 95% confidence interval for CVD mortality still falls into a significant range, the strength of this association requires further modification based on the effective number of subjects. Overall, a long-term follow-up study of subjects at the current level of exposure would improve the strength our results.

Some other factors that might have influenced the arsenic methylation profiles were not considered in this study. It is very difficult to control for the influence of nutritional status and dietary intake. In addition, the distribution of certain rare alleles, including C-108T and L55M in *PON1*, A140D in *GSTO1*, and M287T in *AS3MT*, could not be reliably determined in our sample. The possibility of individual variability in arsenic methylation and its impact on the pathogenesis of CVD cannot be ruled out. Therefore, a further study with a larger sample size with sufficient control for other confounders that may be directly related to arsenic risks is warranted for the evaluation of the relations of genetic variants to abnormal LDH elevation.

In conclusion, this study provides a new perspective of the factors associated with abnormal LDH elevation among subjects with chronic arsenic exposure. **LDH is an arsenic toxicity marker associated with ??*an increased incidence of all-cause and CVD death.** [Dear Author: *Is this what you mean?] **Our findings emphasize the long-term effects of arsenic exposure, which may lead to excessive CVD and physical illness- related deaths.**

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Conflict of interest

The authors have no competing interests or financial disclosures.

References

[Dear Author: You need to use journal abbreviations in your new references.]

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Table 1. The average characteristics, including cardiovascular risk factors, of the subjects in the arsenic-endemic and non-endemic areas in southwestern Taiwan.

Factor	Non-endemic area (Chiali) (N=296)	Endemic area (Putai) (N=380)	P-value
Age (years)	61.5 (10.1)	61.2 (8.9)	0.759
Gender (Male, %)	119 (39.3)	164 (43.3)	0.292
Cigarette smoking (%)	38 (12.8)	80 (21.1)	0.005
Alcohol consumption (%)	26 (8.7)	47 (12.4)	0.122
SBP (mmHg)	138.9 (18.3)	136.9 (22.3)	0.207
DBP (mmHg)	83.8 (10.8)	80.2 (13.8)	<0.001
LDH (IU/L)	**295.1 (73.5)**	**408.4 (224.8)**	**<0.001**
AST (U/L)	22.6 (14.0)	26.4 (16.8)	0.001
ALT (U/L)	15.2 (10.9)	21.7 (21.4)	<0.001
Glucose (mg/dl)	110.6 (30.5)	112.0 (49.8)	0.669
HbA1c (%)	5.9 (1.1)	6.3 (4.7)	0.146
Cholesterol (mg/dl)	201.9 (37.9)	203.0 (37.7)	0.723
Triglyceride (mg/dl)	132.2 (89.3)	137.0 (88.4)	0.488
HDL (mg/dl)	44.7 (12.5)	40.5 (12.7)	<0.001
LDL (mg/dl)	124.9 (36.1)	139.8 (40.3)	<0.001
BMI (kg/m^2)	24.4 (3.2)	25.3 (3.7)	<0.001
Microalbumin (mg/dl)	**3.0 (11.7)**	**5.7 (24.0)**	**0.074**
UA (mg/dl)	7.5 (2.3)	6.6 (3.2)	<0.001

Data are reported as means (S.D) or counts (%)

SBP: systolic blood pressure; DBP: diastolic blood pressure, LDH: lactate dehydrogenase, AST: aspartate aminotransferase; ALT: alanine aminotransferase; HbA1c: glycated hemoglobin A1, HDL: high density lipoprotein; LDL: low density lipoprotein; BMI: Body mass index; UA: uric acid

Table 2. Characteristics of study subjects stratified by LDH levels in arsenic-endemic and non-endemic areas in southwestern Taiwan

	Non-endemic area (Chiali)			Endemic-area (Putai)		
	LDH levels			LDH levels		
Factor	Normal (N=284)	Abnormal (N=12)	P-value	Normal (N=283)	Abnormal (N=97)	P-value
Age (years)	61.1 (10.1)	69.9 (6.16)	0.003	60.6 (9.0)	63.1 (8.3)	0.015
Gender (Male, %)	109 (38.4)	7 (58.3)	0.166	124 (43.82)	40 (41.67)	0.713
Cigarette smoking (%)	36 (12.9)	1 (8.3)	1.000	57 (20.14)	23 (23.71)	0.457
Alcohol consumption (%)	24 (8.54)	1 (8.3)	1.000	36 (12.72)	11 (11.34)	0.722
SBP (mmHg)	138.9 (18.2)	142.2 (20.82)	0.538	135.4 (20.1)	141.2 (22.6)	0.018
DBP (mmHg)	83.8 (10.7)	83.8 (13.04)	0.993	79.4 (13.9)	82.2 (13.5)	0.096
AST (U/L)	21.7 (10.7)	43.5 (42.07)	0.100	23.6 (11.0)	34.6 (25.7)	<0.001
ALT (U/L)	14.8 (9.4)	24.6 (28.60)	0.260	19.6 (16.5)	28.0 (30.9)	0.012
Glucose (mg/dl)	110.37 (30.3)	18.9 (24.20)	0.834	110.0 (49.9)	117.8 (48.8)	0.179
HbA1c (%)	5.9 (1.1)	6.1 (1.64)	0.726	6.3 (5.4)	6.3 (1.7)	0.929
Cholesterol (mg/dl)	202.0 (38.0)	199.8 (37.47)	0.840	204.0 (37.7)	199.8 (37.6)	0.337
Triglyceride (mg/dl)	132.3 (89.3)	129.3 (94.27)	0.908	140.3 (93.7)	127.2 (69.9)	0.207
HDL (mg/dl)	44.6 (12.6)	47.9 (10.66)	0.364	40.3 (13.0)	41.1 (11.7)	0.613
LDL (mg/dl)	125.2 (36.3)	117.2 (34.10)	0.454	141.8 (41.2)	134.0 (37.1)	0.106
BMI (kg/m^2)	24.5 (3.1)	22.6 (4.97)	0.222	25.0 (3.7)	26.3 (3.9)	0.008
Microalbumin (mg/dl)	3.5 (14.9)	1.7 (3.4)	0.668	4.0 (11.4)	6.5 (20.9)	0.079
UA (mg/dl)	7.4 (2.2)	8.6 (3.27)	0.220	6.6 (3.5)	6.5 (2.0)	0.753

Data are reported as means (S.D) or counts (%);

Critical threshold for abnormal LDH elevation was defined as an LDH activity greater than 450 (IU/L); SBP: systolic blood pressure; DBP: diastolic blood pressure, LDH: lactate dehydrogenase, AST: aspartate aminotransferase; ALT: alanine aminotransferase; HbA1c: glycated hemoglobin A1c, HDL: high density lipoprotein; LDL: low density lipoprotein; BMI: Body mass index; UA: uric acid

Table 3. Urinary arsenic species of study subjects stratified by LDH levels in arsenic-endemic and non-endemic areas in southwestern Taiwan

	Non-endemic area (Chiali)			Endemic-area (Putai)		
Factor	Normal (N=279)	Abnormal (N=12)	P-value	Normal (N=256)	Abnormal (N=87)	P-value
As(III) (μg/g creatinine)	2.07 (2.98)	1.82 (3.21)	0.779	3.89 (6.32)	7.20 (12.01)	0.019
As(V) (μg/g creatinine)	2.73 (3.14)	3.26 (3.43)	0.573	2.65 (3.04)	3.55 (8.31)	0.340
iAs (As(III) + As(V))(μg/g creatinine)	4.08 (4.62)	5.08 (5.64)	0.834	6.54 (7.58)	10.75 (15.66)	0.022
MMA (μg/g creatinine)	2.96 (3.12)	4.68 (7.87)	0.465	4.09 (4.13)	5.43 (8.00)	0.149
Σ(iAs +MMA) (μg/g creatinine)	7.76 (6.45)	9.77 (10.28)	0.517	10.63 (9.92)	16.18 (19.82)	0.017
DMA (μg/g creatinine)	42.78 (34.78)	40.94 (22.51)	0.856	34.42 (26.35)	44.76 (49.98)	0.078
Σ(iAs + MMA + DMA) (μg/g creatinine)	50.55 (38.10)	50.70 (31.84)	0.989	45.07 (31.58)	60.94 (60.22)	0.025
PMI (MMA/iAs)	0.84 (0.96)	1.13 (1.57)	0.545	0.84 (0.64)	0.85 (0.90)	0.956
SMI (DMA/MMA)	23.80 (23.10)	21.38 (13.54)	0.570	14.31 (21.53)	22.03 (41.24)	0.098

Data are reported as means (S.D); Critical threshold for abnormal LDH elevation was defined as an LDH activity greater than 450 (IU/L); PMI: Primary methylation index; SMI: Secondary methylation index

Table 4. The association of genotypes in GSTO2, PON1 and PON2 polymorphisms and abnormal LDH elevation in the subjects from arsenic-endemic (Putai) and non-endemic (Chiali) areas in southwestern Taiwan

		Non-endemic area (Chiali)				Endemic area (Putai)			
		LDH level (IU/L)				LDH level (IU/L)			
Gene	SNPs	Normal (N=279)	Abnormal (N=12)	OR (95% CI)	P-value	Normal (N=256)	Abnormal (N=87)	OR (95% CI)	P-value
GSTO2	N142D								
	NN	147	5	1.00 (Reference)		137	41	1.00 (Reference)	
	ND	107	5	1.37 (0.39-4.87)	0.836	94	36	1.28 (0.76-2.15)	0.780
	DD	24	2	2.45 (0.45-13.35)	0.359	17	7	1.38 (0.53-3.55)	0.676
	Missing	1	0	-	-	8	3	-	-
PON1	Q192R								
	RR	104	6	1.00 (Reference)		65	28	1.00 (Reference)	
	QR	104	4	0.67 (0.18-2.43)	0.882	95	30	0.73 (0.40-1.34)	0.378
	QQ	31	1	0.56 (0.07-4.82)	0.907	33	15	1.06 (0.80-2.24)	0.454
	Missing	40	1	-	-	63	14	-	-

PON2	A148G								
	AA	200	9	1.00 (Reference)		121	38	1.00 (Reference)	
	AG	72	3	0.93 (0.24-3.52)	0.952	82	37	1.44 (0.84-2.45)	0.755
	GG	4	0	-	-	7	5	2.28 (0.68-7.59)	0.240
	Missing	3	0	-	-	46	7	-	-
	C311S								
	SS	188	9	1.00 (Reference)		136	46	1.00 (Reference)	
	CS	65	3	0.96 (0.25-3.67)	0.956	62	21	1.00 (0.55-1.82)	0.996
	CC	10	0	0.94 (0.05-17.36)	0.976	4	8	5.91 (1.70-20.55)	0.005
	Missing	16	0	-	-	54	12	-	-

Critical threshold for abnormal LDH elevation was defined as 450 (IU/L)

Table 5. Multivariable association of abnormal LDH elevation among subjects in arsenic-endemic and non-endemic areas in southwestern Taiwan

	Model 1		Model 2	
Arsenic species	OR (95% CI)	P-value	OR (95% CI)	P-value
Age (1-year increment)	1.06 (1.02-1.11)	0.002	1.07 (1.02-1.11)	0.003
Gender (Male vs. Female)	1.44 (0.69-2.99)	0.328	1.43 (0.69-2.96)	0.339
Cigarette smoking (Yes vs. No)	1.16 (0.49-2.74)	0.733	1.15 (0.49-2.71)	0.756
SBP (mmHg)	1.01 (0.99-1.02)	0.390	1.01 (0.99-1.03)	0.337
AST (U/L)	1.06 (1.04-1.09)	<0.001	1.06 (1.04-1.09)	<0.001
HDL (mg/dl)	1.02 (0.99-1.05)	0.076	1.02 (0.99-1.05)	0.142
BMI	1.03 (0.95-1.12)	0.489	1.02 (0.94-1.11)	0.616
Σ(iAs +MMA) (μg/g creatinine)	1.03 (1.01-1.06)	0.010	1.04 (1.01-1.06)	0.009
PON2- C311S				
SS	1.00 (Reference)	-	1.00 (Reference)	-
CS	0.90 (0.45-1.80)	0.143	0.90 (0.45-1.81)	0.144
CC	2.69 (0.67-10.85)	0.120	2.67 (0.67-10.75)	0.203
Duration of arsenic water consumption (years)				
Non-exposed (Chiali)	1.00 (Reference)			
Less than 21 (Putai)	8.65 (3.68-20.34)	0.002		
Greater than 21 (Putai)	9.11 (3.83-21.66)	0.001		
Cumulative arsenic (ppm-years)				
Non-exposed (Chiali)			1.00 (Reference)	
Less than 14.7 (Putai)			8.16 (3.57-18.64)	0.004

Greater than 14.7 (Putai)	9.59 (3.75-24.53)	0.002

Critical threshold for abnormal LDH elevation was defined as 450 (IU/L)

Table 6. The association of abnormal LDH elevation with mortality among subjects in arsenic-endemic area by cox proportional hazards analysis

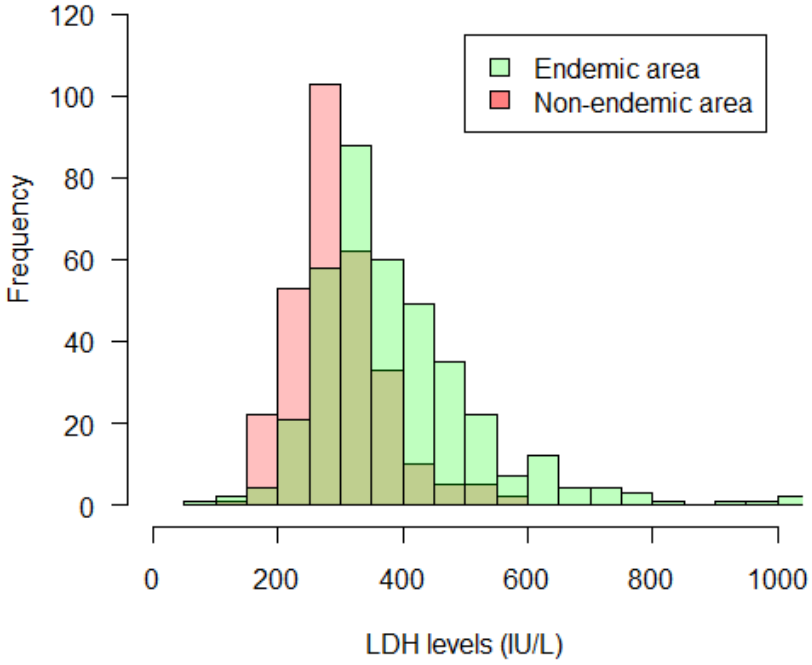
	All-cause mortality[a]		CVD mortality	
Arsenic species	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**
Abnormal LDH elevation	**3.07 (1.41-6.69)**	**0.005**	**6.56 (1.39-31.07)**	**0.017**
Framingham risk score for CVD	**1.05 (0.96-1.14)**	**0.296**	**1.16 (0.99-1.14)**	**0.112**
Cumulative arsenic (ppm-years)				
Less than 14.7 (Putai)	**1.0 (Reference)**		**1.0 (Reference)**	
Greater than 14.7 (Putai)	**1.09 (1.06-1.13)**	**<0.001**	**1.07 (0.99-1.14)**	**0.082**
PON2- C311S				
SS	**1.0 (Reference)**		**-**	
CS	**0.63 (0.26-1.55)**	**0.314**	**-**	
CC	**3.77 (0.78-18.24)**	**0.099**	**-**	

Critical threshold for abnormal LDH elevation was defined as 450 (IU/L);

Mortality data was calculated from the date of study entry in 2002 to year 2009 according to ICD-9

[a]Excluding 2 accidental and homicide deaths which are not from conventional natural-causes

1
2



Figure

Histogram of the LDH levels of subjects from arsenic-endemic and non-endemic areas in southwestern Taiwan

[Dear Author: If you have only one figure, you don't need to number it.]