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Arsenic Exposure and Circulating Uric Acid Levels: A Population Based Cross-Sectional Study in Bangladesh

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ARSENIC EXPOSURE AND CIRCULATING URIC ACID LEVELS: A POPULATION BASED CROSS-SECTIONAL STUDY IN BANGLADESH

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Certificate

I certify that the thesis entitled "**ARSENIC EXPOSURE AND CIRCULATING URIC ACID LEVELS: A POPULATION BASED CROSS-SECTIONAL STUDY IN BANGLADESH"** submitted by Md. Nazmul Huda, incorporates the original research work carried out by him in my laboratory, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh under my supervision. I am forwarding his thesis being submitted for the award of the degree of Doctor of Philosophy of the University of Rajshahi. This work has not been submitted previously anywhere for the awards of any degree.

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Declaration

I do hereby declare that the materials embodied in this entitle "**ARSENIC EXPOSURE AND CIRCULATING URIC ACID LEVELS: A POPULATION BASED CROSS-SECTIONAL STUDY IN BANGLADESH"** prepared for submission in the Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh, for the degree of Doctor of Philosophy, are original research works of mine and have not been previously submitted for the awards of any degree anywhere.

(Md. Nazmul Huda)

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Abstract

Blood uric acid has been recognized as a putative marker for cardiovascular diseases (CVDs). CVDs are the major causes of arsenic-related morbidity and mortality. However, association of arsenic exposure with plasma uric acid (PUA) levels in relation to CVDs has not yet been explored. Therefore, this study was designed to investigate the PUA levels and its relations to the hypertension and circulating biomarkers of CVDs recruiting human subjects from arsenic-endemic and non-endemic rural areas in Bangladesh. A total of 483 subjects, 322 from arsenic-endemic and 161 from non-endemic areas in Bangladesh were recruited as study subjects. The male and female study subjects in arsenic-endemic areas were 168 and 154 respectively, whereas these were, 75 and 86 respectively, in non-endemic area. Arsenic concentrations in the drinking water, hair and nails of the study subjects were measured by inductively coupled plasma mass spectroscopy (ICP-MS) with appropriate certified reference material (CRM). PUA levels were measured using a colorimetric method. The average PUA levels (Mean ± SE) of male study subjects in arsenic-endemic and non-endemic areas were 4.95 ± 0.96 and 4.11 ± 0.74 mg/dl respectively, whereas these were 4.29 ± 0.86 and 3.55 ± 0.84 mg/dl respectively, of female study subjects in arsenic-endemic and non-endemic areas. The differences of PUA levels in both sexes in arsenic-endemic and non-endemic areas were statistically significant ($p < 0.001$). Arsenic exposure (water, hair and nail arsenic concentrations) showed significant positive correlations with PUA levels. In multiple regression analyses, arsenic exposure levels were found to be the most significant contributors on PUA levels among the other variables that included age, body mass index, blood urea nitrogen (BUN) and smoking. There were dose-response relationships between

arsenic exposure and PUA levels. Furthermore, diastolic blood pressure (DBP) and systolic blood pressure (SBP) showed significant positive correlations with PUA levels. The average PUA levels were significantly higher in the hypertensive group than those in the normotensive group in both males and females living in arsenic-endemic areas. Finally, it was observed that PUA levels had significant positive associations with C-reactive protein (CRP), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Taken together, the results of this study suggested that arsenic exposure-related elevation of PUA levels might be implicated in arsenic-induced CVDs.

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LIST OF ABBREVIATIONS

PUA	Plasma Uric Acid
BUN	Blood Urea Nitrogen
MMA	Monomethylarsonic Acid
DMA	Dimethylarsinic Acid
ANOVA	One-way Analysis of Variance
As	Arsenic
AS3MT	Arsenic methyltransferase
LDL	Low Density Lipoprotein
SPSS	Statistical Package for Social Sciences
ROS	Reactive Oxygen Species
WHO	World Health Organization
SOES	School of Environmental Studies
DPHE	Department of Public Health Engineering
BMI	Body Mass Index
NRC	National Research Council
IPCS	International Programme on Chemical Safety
ICP-MS	Inductively Coupled Plasma-Mass Spectroscopy
CRM	Certified Reference Material
DBP	Diastolic Blood Pressure
EPA	Environmental Protection Agency
BGS	British Geological Survey
USA	United States of America
UNICEF	United Nations International Children's Emergency Fund
ICAM-1	Intercellular Adhesion Molecule-1
VCAM-1	Vascular Cell Adhesion Molecule-1
CVDs	Cardiovascular Diseases
NO	Nitric Oxide
et al.	With Others
g	Gram
dl	Decilitre

L	Litre
SL	Serial
No./no.	Number
µg	Microgram
SD	Standard Deviation
SE	Standard Error

Chapter 1

Introduction

Introduction

1.1 General background

Chronic exposure to arsenic is a major threat to the public health in many countries in the world. Arsenic is ubiquitously found in environment. It is present in food, soil and water. It is released in the environment from both natural and man-made sources (Tchounwou et al., 1999; Roy and Saha, 2002). Exposure to higher than average level of arsenic occurs either in workplace, e.g., in smelting industries, coal fired power plants, cosmetic industries, agriculture, or through arsenic contaminated food and drinking water. The most common forms of arsenic are water-soluble arsenite (the trivalent form, As III) and arsenate (the pentavalent form, As V). Trivalent arsenic is more toxic than the pentavalent arsenic and its inorganic forms are more toxic than organic forms (Bertolero et al., 1987). Chronic exposure to arsenic has been reported to be associated with a variety of cancers, cardiovascular diseases, diabetes mellitus, dermatitis, immunotoxicity, peripheral neuropathy, liver dysfunction and many other complications (Tapio and Grosche., 2006; Mumford et al., 2007; Chen et al., 2007; Mazumder et al., 1998; Cheng et al., 2004; Meliker et al., 2007; Vahidnia et al., 2008; Wang et al., 2002). Since decades, chronic arsenic toxicity is a widespread global problem affecting millions of people all over the world. Bangladesh is one of the most severely arsenic affected countries in the world. In Bangladesh, arsenic poisoning has not only created human sufferings and death but also become socio-economic burden to the country. It has been reported that 61 districts out of 64 and about 60% of the land area of Bangladesh are affected by arsenic contamination (SOES, DCH, 2000), and people of these areas are consuming water at greater than the permissive limit (<10 μg/L) set by World Health Organization (WHO). Arsenic contamination of

ground water poses a serious threat to the agricultural sustainability of the country. Besides domestic use, significant quantities of water from shallow aquifers are being used in the dry season particularly for irrigating rice and others crops in Bangladesh. The dependency of ground water for drinking, cooking and irrigation resulting in a large quantity of arsenic is being cycled through environment each year with a major implication on public health and environment.

1.2 Physico-chemical properties of arsenic

Arsenic is regarded as metalloid i.e., it has properties of both metals and non-metals. It is a group V element with symbol As, atomic number 33 and molecular weight 74.92. Arsenic primarily exists at four different valence states +5, +3, 0 and –3. Elemental arsenic has a valence state of 0. Arsine and arsenides have a valence of –3. Arsenic is found to exist in combination with oxygen, hydrogen, chlorine, sulphur, different metals, and also as a pure elemental crystal. Arsenic can exist as powder, amorphous or vitreous forms. Elemental arsenic has a specific gravity of 5.73, sublimes at 613°C and has a very low vapour pressure of 1 mm Hg at 373°C. Many of the inorganic arsenic compounds occur as white, odorless solids with specific gravities ranging from about 1.9 to more than 5. Elemental arsenic is insoluble in water. Both organic and inorganic arsenic species can dissolve in water. Monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) are the major form of organic arsenic (Tamaki and Frankenberger, 1992). Inorganic forms of arsenic are more toxic than organic form. The trivalent form (As^{III}, called arsenite) and the pentavalent form (As^{V}, called arsenate) of inorganic arsenic are the principal inorganic species which tend to be more prevalent in water than the organic arsenic species (Ferguson and Gavis, 1972). Under reducing conditions arsenite is the

dominant form; whereas arsenate is generally the more stable form in oxygenized environments (NRC, 1999). When heated to decompose, arsenic compounds emit toxic arsenic fumes (HSDB, 2003). Arsenic trioxide, the most common arsenic compound in commerce, melts at 312°C and boils at 465°C. Arsenic does react with hot acids to form arsenous acid (H_3AsO_3) or arsenic acid (H_3AsO_4).

Table 1.1 Chemical nature of arsenic at a glance

Atomic number	33
Atomic mass	74.922 g/mol
Electro negativity according to Pauling	2.0
Density	5.7 g/cm^3 at 14°C
Melting point	814 °C (36 atm)
Boiling point	615 °C (sublimation)
Atomic radius	0.139 nm
Oxidation states	-3, +3, +5
Key isotope	^{75}As
Electronic shell	[Ar] 3d^{10} 4s^2 4p^3
Energy of first ionization	947 kJ/mol
Energy of second ionization	1798 kJ/mol
Energy of third ionization	2736 kJ/mol
Standard potential	- 0.3 V (As^{3+}/ As)
Discovered by	Albertus Magnus

Source: [Lenntech, Netherlands: Alumni from the Technical University of Delft. Available at: http://www.lenntech.com/periodic/elements/as.htm]

1.3 Occurrence and exposure route of arsenic

Arsenic is a naturally occurring element found throughout the environment. It is released into the environment from both natural and man-made sources. It occurs in more than 245 minerals. It ranks 20th in natural abundance, 14th in seawater and 12th in the human body (Mandal and Suzuki, 2002). People are exposed to arsenic through water, food, air, occupation and other sources. It has widespread use in agriculture, medicine, ceramic and electrical industries (Nriagu and Azcue, 1990). The sources from where arsenic enters into environment are mentioned bellow:

1.3.1 Natural sources of environmental arsenic

Arsenic is distributed ubiquitously throughout in the earth crusts, soil, sediments, water, air and living organisms. The major sources of naturally occurring arsenic are as follows.

1.3.1.1 Earth crusts

Arsenic is most abundantly present in earth crust and the average concentration of arsenic in different types of rock (igneous rocks: a crystalline solids which form directly from the cooling of magma and sedimentary rocks: a thin veneer of loose sediment that cover the earth crust mostly composed of igneous rock) is 2 mg/kg. Arsenic occurs from 0.5 to 2.5 mg/kg in most rocks, and higher concentrations were found in finer-grained argillaceous sediments and phosphorites (Kabata-Pendias and Pendias, 1984). Arsenic is concentrated in some reducing marine sediment especially those associated with gold mineralization up to 3000 mg/kg. It is also concentrated with iron hydroxides and sulfides in sedimentary rocks. Iron deposits, sedimentary iron ores and manganese nodules were rich in arsenic. Arsenic naturally occurs in over 200 different mineral forms, of which approximately 60% are arsenates, 20%

sulfides and sulfosalts and the remaining 20% includes arsenides, arsenites, oxides, silicates and elemental arsenic (Onishi, 1969). Arsenic in its most recoverable form is found in various types of metalliferous deposits. The major deposits of this type are categorized into seven major groups. It is common in iron pyrite, galena, and chalcopyrite and less common in sphalerite (Goldschmidt, 1954). The most common arsenic mineral is arsenopyrite.

1.3.1.2 Soil and sediment

Arsenic is found in the soils of various countries range from 0.1 to 50 mg/kg and mean 5 mg/kg, but varies considerably among geographic regions (Colbourn et al., 1975; Garelick et al., 2008; Mandal and Suzuki, 2002; Vinogradov, 1959). Concentrations of arsenic in soils are higher than those in rocks and lowest in sandy soils and those derived from granites, whereas larger concentrations are found in alluvial and organic soils (Kabata-Pendias and Pendias, 1984; Peterson et al., 1981). Arsenic concentrations in uncontaminated soil are generally in the range between 0.2–40 mg/kg (WHO, 1981). The average arsenic content in agricultural land in Bangladesh varied from 4 to 8 mg/kg but it rose up to 83 mg/kg due to continuous use of contaminated irrigation water (Ullah, 1998). Arsenic and its compounds are mobile in the environment. Weathering of rocks converts arsenic sulfides to arsenic trioxide, which enters the arsenic cycle as dust or by dissolution in rain, rivers or groundwater.

1.3.1.3 Water

Groundwater is the major source of arsenic and humans are generally exposed to arsenic through contaminated drinking water. Arsenic can enter into water supplies from natural deposits which then enter into the food chain, causing widespread distribution throughout the plant and animal kingdoms. Inorganic species of arsenic is

more dominant in water. Under oxidation state pentavalent (As^{V}) form is more dominant, whereas in reduced environment trivalent (As^{III}) form of inorganic arsenic is more stable.

1.3.1.4 Food

Food is another important source of human exposure to arsenic. Rice is staple food in many areas of the world (Meharg and Zhao, 2012). Rice and vegetables were found to contain higher amount of arsenic in arsenic-affected areas like Bangladesh because of excessive use of arsenic-contaminated groundwater for irrigation (Duxbury et al., 2003; Meharg and Rahman, 2003). Toxic inorganic arsenic is present relatively high proportion in rice with increased bioavailabilities and bioaccessibilities (Juhasz et al., 2006; Meharg and Raab, 2010; Trenary et al., 2012). Organic arsenic such as arsenobetaine, arsenocholine, arsenosugars, tetramethylarsonium salts, and arsenic-containing lipids have been found in high amount in seafood and marine organisms, although some of these compounds have also been found in terrestrial species (Francesconi and Edmonds, 1997; Grotti et al., 2008). These arsenic derivatives are not acutely toxic because of their low biological reactivity and their rapid excretion in urine. Arsenic concentrations in seafood amount 2.4–16.7 mg/kg in marine fish, 3.5 mg/kg in mussels and more than 100 mg/kg in certain crustaceans (Buchet et al., 1984; Ishinishi et al., 1977). Appreciable amount of trivalent inorganic arsenic have been observed in wine made from grapes on which arsenic containing pesticide sprayed (Hughes et al., 1994). The amount of ingested arsenic via foods mainly depends on the seafood in the diet especially where seafood consumption rate is very high. It has been reported that Japanese population intake more amount of arsenic than in Europe and the United States because of their diet contain larger amount of

seafood. The diet in Japan was found to contain 5.7–17% inorganic arsenic, 1.1–3.6% monomethylarsonate (MMA), 6.6–27% dimethylarsinate (DMA) and 47.9–75.2% arsenobetaine (Yamauchi and Fowler, 1994).

1.3.1.5 Air

In the atmosphere, arsenic exists as particulate matter, mostly less than 2 μm in diameter. It is usually present in air as a mixture of arsenite and arsenate. People are generally exposed to arsenic through air is very low and normally arsenic concentrations in air ranges from 0.4 to 30 ng/m^3 and less than 1% of total arsenic exposure (WHO, 1996). According to US EPA the estimated average national exposure in the U.S. is at 6 ng/m^3 arsenic. The amount of arsenic inhaled per day is about 50 ng or less (assuming that about 20 m^3 of air is inhaled per day) in unpolluted areas (WHO, 1981). The daily respiratory intake of arsenic is approximately 120 ng of which 30 ng would be absorbed (Zuane, 1990). Typical arsenic levels for the European region are currently quoted as being between 0.2 and 1.5 ng/m^3 in rural areas, 0.5 and 3 ng/m^3 in urban areas and no more than 50 ng/m^3 in industrial areas (DG Environment, 2000).

1.3.2 Anthropogenic source of arsenic

Elemental arsenic is produced commercially from arsenic trioxide. Arsenic trioxide is a by-product of metal smelting operations. About 70% of the world production of arsenic is used in timber treatment, 22% in agricultural chemicals and the remainder in glass, pharmaceuticals and metallic alloys [IPCS (International Programme on Chemical Safety)]. Mining, metal smelting and burning of fossil fuels are the major industrial processes that contribute to arsenic contamination of air, water and soil. The use of arsenic-containing pesticides in the past has left large areas of agricultural land

contaminated. The use of arsenic in the preservation of timber has also led to contamination of the environment. In addition, the use of arsenic-contaminated groundwater for irrigation leads to widespread contamination of land and additional exposure to human and livestock via food all over the world (Kile et al., 2007; Lindberg et al., 2007; Meharg and Rahman, 2003).

1.4 Environmental transport and distribution of arsenic

In the atmosphere, arsenic is emitted by high-temperature processes such as coal-fired power generation plants, burning vegetation and volcanism. Primarily, arsenic in the atmosphere is released as As_2O_3 and exists mainly adsorbed on particulate matter. These particles are then dispersed through wind and returned to the earth by wet or dry deposition.

In soils or sediments arsines released from microbial sources undergo oxidation in the air and reconverting the arsenic to non-volatile forms that settle back to the ground. The water soluble forms of arsenic include arsenate, arsenite, methylarsonic acid (MMA) and dimethylarsenic acid (DMA). Almost all arsenic present in well-oxygenated water and sediments is in more stable pentavalent state (arsenate). Depending on the redox potential, pH and biological processes, some arsenic species can interchange their oxidation state. There is also affinity of some arsenic species for clay mineral surfaces and organic matter which lead to affect their environmental behavior. Weathered rock and soil may be transported by wind or water erosion. Many arsenic compounds tend to adsorb to soils and leaching usually results in transportation over only short distances in soil. There are three major modes of arsenic biotransformation in arsenic cycle found to occur in the environment: redox

transformation between arsenite and arsenate, the reduction and methylation of arsenic, and the biosynthesis of organoarsenic compounds represented in Figure 1.1.

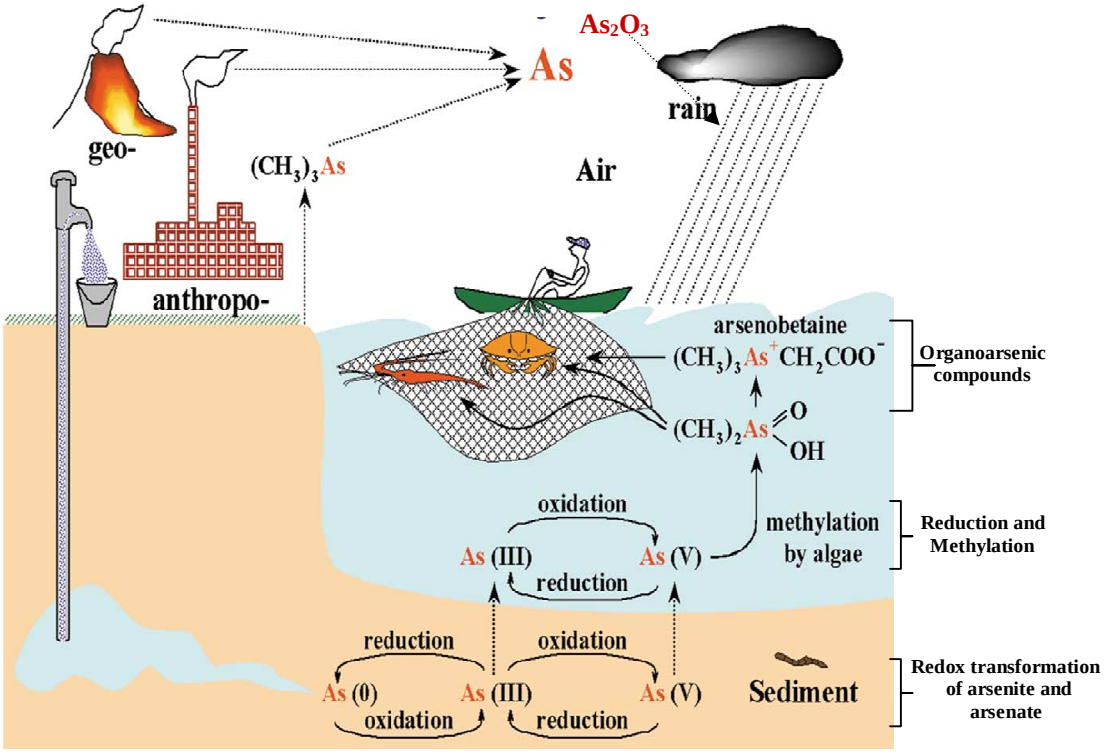


Figure 1.1 Bio-transformation of arsenic in environment through arsenic cycle
(Source: Center for Computational Physics University of Coimbra)
Available at: http://www.viveraciencia.org/

1.5 Recommended value of arsenic in drinking water set by WHO

According to WHO, arsenic is one of 10 chemicals of major public health concern. The toxic effects of arsenic depend on the nature and extent of exposure, particularly the frequency of exposure, duration of exposure and type of arsenic present. A lot of efforts were given by WHO to reduce arsenic poisoning includes setting guideline values, reviewing evidence and providing risk management recommendations. WHO publishes a guideline value for arsenic in its Guidelines for Drinking-Water Quality. WHO established 200 µg/L as an allowable concentration in the first version of

International Standards for Drinking-Water in 1958. In 1963, in the second version a stricter concentration of 50 µg/L arsenic was set as a new standard. In the last edition of the WHO Guidelines for Drinking-Water Quality published in 1993, a further stricter standard of 10µg/L arsenic was suggested. In 2001, EPA in the United States adopted a reduced standard of 10µg/L for public water supplies. However, highly contaminated countries of the world such as Bangladesh and India have set up their standards 5 times higher than the maximum permissive limit set by WHO. Thus the maximum permissive limit of arsenic in drinking water set by Bangladesh Government is 50µg/L. The intention of the guidelines is to be used as the basis for regulation and standard setting worldwide, for the development of national standards that, if properly implemented, will ensure the safety of drinking water supplies through the elimination, or reduction to a minimum concentration, of constituents in drinking water that are known to be hazardous to public health. The standard value of arsenic in guideline was designated as provisional due to the measurement difficulties and practical difficulties in removing arsenic from drinking water. It is when difficult to achieve the guideline value, member countries may set higher values as standards taking into account local circumstances, resources and risks.

1.6 Global scenario of arsenic contamination

Arsenic contamination is a cause of great concern in many countries of the world. Arsenic poisoning has been reported in many countries including Argentina, Bangladesh, Chile, China, India (West Bengal), Mexico, Nepal, Pakistan, Vietnam, Japan and the USA. Arsenic pollution in different countries is described briefly here.

Argentina: Groundwater in the central part of Argentina contains arsenic concentrations that, in most cases, exceed the value suggested by World Health

Organization. In this region, quaternary loessical sediments with a very high volcanic glass fraction lixiviate arsenic and fluoride after weathering (Francisca et al., 2009). Cutaneous disorders due to the chronic poisoning of arsenic were known in Argentina as 'Bell wills Disease.

Mexico: The first information on arsenic contamination was reported in 1962, when 40 serious cases and one death were found in the urban area of Torreon, Coahuila in Mexico (M.L. Castro de Esparza, International Congress Mexico City, 20-24 June, 2006). The excessive amount of arsenic in drinking water was found in the Durango, Coahuila, Zacatecas, Morelos, Aguas Calientes, Chihuahua, Puebla, Nuevo Leon, Guanajuato, San Luis Potosi and Sonora aquifers and the Lagunera region (Finkelman et al. 1993; Avilés & Pardón, 2000). It is estimated that around 450,000 people are exposed to arsenic in Mexico.

Chile: Arsenic contamination in water was first detected in 1957 in a province named Antofagasta (northern Chile) in Chile. Antofagasta province in Chile has a well-documented history of arsenic exposure from naturally contaminated water. Not only Antofagasta, arsenic contamination was also found in other cities in Chile such as Calama, Santiago, Rancagua, Taltal, Tocopilla and San Pedro de Atacama. Approximately 500,000 inhabitants were exposed to arsenic contamination (M.L. Castro de Esparza, International Congress Mexico City, 20-24 June, 2006). Almost all the diseases caused by arsenic were found in human through various researches in Chile. Many people embraced death due to drinking of arsenic-contaminated water since 1957. It was estimated that arsenic might account for 7% of all deaths among those aged 30 years and over from 1989 to 1993 (Smith et al., 1998).

USA: Relatively low level of arsenic was found in groundwater in the USA. About 3 million people of the country are drinking of arsenic contaminated water. Widespread

high concentrations of arsenic were found in the West, the Midwest, Parts of Texas, and Northeast in the USA (Mapping arsenic in groundwater).

Taiwan: The arsenic contamination in Taiwan was reported since 1968. A disease called 'black foot disease' spread in the county massively. Later it was known that the cause of the disease was arsenic received through contaminated tube-well water. Water treatment plants were established in 1956. But every year new arsenic-affected patients are found as they drank arsenic contaminated water earlier. At present, some 1,00,000 people are suffering from arsenic problem in Taiwan.

Mongolia: The first arsenic-affected patient was found in Mongolia in 1988. A survey conducted in 1989 revealed that some 3,00,000 people of 627 villages of 11 countries were taking arsenic contaminated water. Among them, 1774 were identified as arsenic-affected patients. Arsenic concentration above 0.05 mg/L was detected in 90% tube-wells of Mongolia. It was thought that the problems arose due to continuous irrigation for agriculture by ground water in this region. Several steps have been taken to ensure supply of safe drinking water in the county. However, satisfactory solution of this problem is very difficult due to financial constraints and affected areas are widely scattered over a vast land. So there is a long way to go before a satisfactory solution is reached.

Philippines: Presence of arsenic was found in human bodies living of both sides of Matingao and Marbol rivers after a geothermal power plant was set up in 1992. The Geothermal plant is suspected as the cause of arsenic contamination. The state-own Philippine national oil company, which installed the plant, has taken measures such as the installation of small water-supply system at various points of the affected area. A total of 39 arsenic patients were detected in the affected area in September 1995.

China: First arsenic patient was found in a province of China in 1953. Medical surveys began in 1964. In 1992, about 3,000 arsenic patients were identified. Another survey conducted in 1991 to 1993 examined 9,202 people and found 1,545 people suffering from arsenicosis. Of them, 88% received arsenic from foods while 7% from water and 5 % from water and 5% from air. In China, use of coal as fuel is considered as the main reason of arsenic contamination. Some coal contains high levels of arsenic in the country.

Japan: Japan experienced several incidents of arsenic exposure. The major incidents include arsenic poisoning in milk, soy sauce, and well water, pollution originating from the Toroku Mine on the island of Kyushu, the Matsuo Mine in Shimane Prefecture and Saganoseki Smelter on Kyushu (Tsuchiya, 1977). In 1955, arsenic affected children were indentified. Children were exposed to arsenic through Morinaga dried milk that contained excessive amount of arsenic (Dakeishi et al., 2006). Arsenic contamination was detected in water and air of two villages-Turoko and Matsu. It was proved that the two villages were affected by arsenic for 50 years. A total of 217 arsenic patients were found in the two villages in 1995. Metal and coalmines were the sources of arsenic pollution in the villages.

India: West Bengal is the most severely affected state of India. High levels of arsenic were detected in the ground water of 8 districts of West Bengal. As a result, 40 million people of the districts covering 38,000 square kilometers are now at risk. School of Environmental Studies (SOES) of Jadavpur University in Calcutta conducted a survey in the affected areas. It identified 863 villages of the 8 districts where ground water contains arsenic more than maximum permissible limit (0.05 mg/l). According to the SOES, 1.5 million people are taking high arsenic containing ground water in the affected area in West Bengal and the major affected districts are -

Malda, Murshidabad, Nadia, Bardhawan, North and South 24 Pargans, Hawra and Hoogli.

Figure 1.2 Arsenic in groundwater across the world
(Available at: http://www.sickkids.ca/PGPR/Symposia-and-Workshops/Oct-2007-china/arsenic-pollution/index.html)

1.7 Arsenic contamination: Bangladesh perspective

Arsenic poisoning was first identified in Bangladesh in 1993 by the Department of Public Health Engineering (DPHE) in the Chapai Nawabganj district (Khan et al., 1997). But the fact remained behind the screen till 1996. Further testing was done in the following years; this included investigations by the Department of Occupational

and Environmental Health of the National Institute of Preventive and Social Medicine. Results from various laboratories were collected in a WHO country situation report in 1996 (WHO, 1996a). The institutions that provided results included the Jadavpur University in Calcutta, India, the Bangladesh Atomic Energy Commission, the Department of Public Health Engineering's laboratories in the Khulna and Rajshahi districts, and the National Institute of Preventive and Social Medicine in Dhaka. Altogether, 400 measurements were presented in the report, although contamination in some wells was measured by more than one laboratory. In about half of the measurements, concentrations were above 50 µg/L (WHO, 1996a) which is clearly in excess of the maximum level recommended by WHO of 10 µg/L (WHO, 1996b) and greater than the maximum level of 50 µg/l permitted in Bangladesh (UNICEF, 1999). On the basis of above results it has been confirmed that the ground water in Bangladesh is severely contaminated by arsenic. The millions of shallow, deep wells and hand operated tube wells that had been sunk in various parts of the country for irrigation and drinking water purposes are now the major source of arsenic contamination. In a consequence, a large number of populations in Bangladesh are suffering from arsenic toxicity. Many people have already died of chronic arsenic exposure related diseases. Arsenic polluted areas in Bangladesh are shown in Figure 1.3.

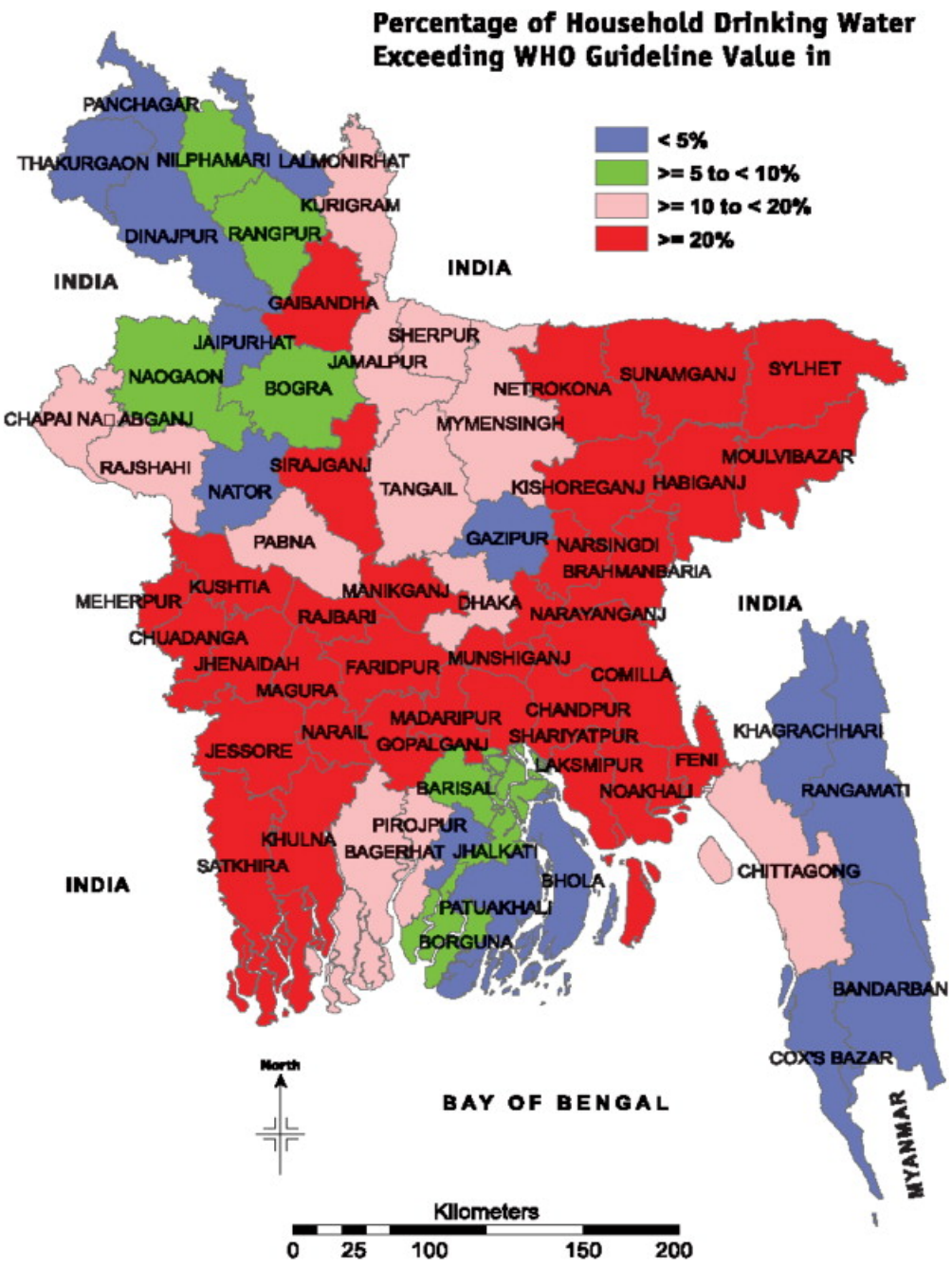


Figure 1.3 Arsenic polluted areas in Bangladesh. Arsenic contamination of household drinking water in Bangladesh in 2009 depicted by the Bangladesh Bureau of Statistics (BBS) and UNICEF Multiple Indicator Cluster Survey data.

1.8 Causes of arsenic pollution in Bangladesh

The major causes of arsenic toxicity in Bangladesh are from contamination of drinking water from natural geological sources rather than from mining, smelting or agricultural sources such as pesticides or fertilizers (Matschullat, 2000). It is generally believed that the source of excessive arsenic in ground water of Bangladesh is geological (BGS, 2000). There are two hypotheses for groundwater arsenic contamination.

Pyrite oxidation: The water flow of many trans-boundary rivers in Bangladesh have been significantly decreased due to the withdrawal of water by upstream country India. Therefore, dependency of ground water has been increased. During the last few decades, the demand of ground water for domestic uses and irrigation purposes increased dramatically. Due to heavy ground water withdrawal the aquifer becomes aerated that allows the oxidation of the pyrite (FeS2) rich in arsenic, with concomitant release of soluble arsenic into the groundwater (Das et al., 1995, 1996; Chowdhury et al., 2000).

Oxy-hydroxide reduction: Another group of scientists (Ahmed et al., 2004; Bhattacharya et al., 1998; Nickson et al., 2000) believe that arsenic is derived by desorption from iron or manganese ox-hydroxide minerals present as a dispersed phase (e.g. as a coating) on the aquifer sediments under reducing conditions. There is little or no sulphides in groundwater in Bangladesh, therefore, it is increasingly accepted that the second concept is most likely explanation of groundwater pollution with arsenic in Bangladesh (WHO, 2000; UNICEF, 1999).

1.9 Metabolism of arsenic

The liver is the primary organ for the metabolism of arsenic compound. The absorption of arsenic into the blood stream occurs at the cellular level and is taken up by red blood cells, white blood cells and other cells that can reduce arsenate to arsenite (Winski and Carter, 1995). Before methylation arsenate is reduced to arsenite form (Miller et al., 2002; Vahter, 2002; Vahter and Marafante, 1983). The primary metabolic step of inorganic arsenic in human is its methylation in the liver. The methylation of arsenic has been demonstrated by the presence of monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in the urine and bile (Cui et al., 2004; Li et al., 2008). Arsenic (III) is methylated in the liver by arsenic methyltransferase (AS3MT) to generate monomethylarsonic acid (MMA) which is reduced to monomethylarsonous acid (MMA) and then further methylated to dimethylarsinic acid (DMA), followed by reduction to dimethylarsinous acid. In both step of methylation, S-adenosyl methionine provides methyl group. These metabolites are more readily excreted through urine. Some other less important routes of elimination of arsenic include feces, skin, sweat, hair and nails. Humans excrete a mixture of inorganic, monomethylated and dimethylated forms of arsenic. The pentavalent metabolites MMA (V) and DMA (V) are believed to be less toxic than arsenite or arsenate (Marafante and Vahter, 1987). Inorganic arsenic and its methylated metabolites are mostly excreted through urine in 2-4 days. Approximately 50% of excreted arsenic in human urine is dimethylated and 25% is monomethylated, rest of them is inorganic (Buchet et al., 1981). Some studies related to arsenic metabolism have also suggested that methylation of inorganic arsenic may be a toxification, rather than a detoxification pathway since the trivalent methylated arsenic metabolites, particularly monomethylarsonous acid (MMA III) and dimethylarsinous acid (DMA

III) are unusually capable of interacting with cellular targets such as proteins and DNA (Goering et al., 1999; Kitchin, 2001). Methylation capacity in human appears to decrease at high arsenic doses. The type and patterns of methylated arsenic species in urine are similar between siblings and between siblings and parents that indicates that arsenic methylation is genetically linked (Chung et al., 2002). When the methylating capacity of the liver is exceeded, exposure to excess levels of inorganic arsenic results in increased retention of arsenic in soft tissues. Recent report has indicated that methylation capacity of arsenic is higher in females than males (Wei et al., 2016).

1.10 Signs and symptoms of arsenicosis

Arsenicosis is chronic health condition resulting from prolonged ingestion (not less than 6 months) of arsenic above a safe dose, usually manifested by characteristic skin lesions, with or without involvement of internal organs. Chronic exposure to increased levels of arsenic affects human health in many ways and manifests its signs and symptoms. Hallmark feature of chronic arsenic exposure include keratosis of the palms and soles, and hyper pigmentation of the limbs (Mazumder et al., 1998). Skin lesions usually develop after 5-10 years of exposure but shorter latencies are also possible. Long-term exposure to arsenic via contaminated drinking water can cause both acute and chronic toxic effects in all the organs and systems of the human body: skin, nervous system, liver, cardiovascular system, endocrine and respiratory system. Acute toxicity may occur in short duration but chronic arsenicosis take longer period and may not become apparent clinically for a decade or more (Maidul et al., 1996). Severity of arsenic toxicity depends on chemical and physical form of arsenic compound, the route by which it enters the body, the dose and duration of exposure, dietary levels of interacting elements, age and sex of the individual (Khan et al.,

1997). Symptoms of acute toxicity are severe projectile vomiting and watery diarrhea, muscular cramp, facial edema, and cardiac abnormalities (Khan et al., 1997; WHO, 1981). Chronic arsenic poisoning is associated with brown pigmentation and keratosis of palm, sole and rarely in the body along with other signs like anorexia, lethargy, diarrhoea or constipation, anemia, abdominal pain, neuropathy and so on (Bakshi, 1968; Hoque et al., 1996). Melanosis (spotted or diffuse), keratosis of palm and sole (spotted or diffuse), leukomelanosis (rain drop pigmentation), and hyperkeratosis are predominant skin symptoms of arsenicosis patients in Bangladesh.

1.11 Biomarkers of arsenic

A biomarker is defined as a xenobiotically induced alteration in cellular or biochemical components or processes, structures or functions, which is measurable in a biological system or sample accurately and reproductively. Biomarkers are classified as those of exposure, effect, and susceptibility. In toxicological point of view selection of appropriate biomarkers to be used for risk assessment is very much important and selection of biomarkers is based on the mechanism of a chemically induced disease state (Goyer, 1996). Arsenic is ubiquitously present in the environment. Contaminated drinking water and foods are the principal routes of human exposure to arsenic. After absorption arsenic is rapidly distributed throughout the body via blood and highest concentration is accumulated in skin, nails and hair. In epidemiology several biomarkers have been identified and used as arsenic exposure markers. Blood, urine, hair, nails are the most commonly used biological samples for different assays studying health risk of arsenic exposure.

Blood: Blood arsenic levels are best suited to evaluate the recent exposure to high dose of arsenic exposures. Speciation of blood arsenic has been used as a biomarker

of arsenic exposure in many epidemiological studies using plasma, serum, and hemolyzed blood (Bemramdane et al., 1999; Ebdon et al., 1999; Huang et al., 2008; Mandal et al., 2004; Shibata et al., 1994; Slejkovec et al., 2008). Quantification of arsenic in the blood can be useful but it depends on the kind of epidemiological study. To study the dose–response relationship of individuals with ongoing chronic exposure and over a wide range of arsenic exposures are best by analyzing total blood arsenic concentrations (Hall et al., 2006; Mandal et al., 2004). Recent seafood ingestion by the subjects may show interference in the analysis of total arsenic in blood (Hughes, 2006). In addition, blood samples are more difficult to obtain for epidemiological research because the sampling is invasive. So blood arsenic is not an ideal exposure biomarker but investigation of blood samples is important to understand the biological effect of chronic exposure to arsenic (Marchiset-Ferlay et al., 2012).

Urine: Urinary arsenic has been used as a biomarker mostly in the epidemiological studies. Total arsenic in urine has often been used as an indicator of recent arsenic exposure because most of the arsenic species are excreted from the body through urine. Many epidemiological studies demonstrated the correlation between water arsenic and urinary arsenic concentrations (Calderon et al., 1999; Lindberg et al., 2006; Mandal et al., 2001, 2004; Meza et al., 2004; Sun et al., 2007). Total arsenic as well as arsenic species [inorganic arsenic (iAs), methylarsonic acid (MMAV) and dimethylarsinic acid (DMAV)] have been measured from human urine samples in several studies. Urinary arsenic species are better indicator for assessing the health risk of arsenic toxicity and methylation capacity of human body (Chen et al., 2005; Tseng, 2007; Vahter et al., 1999). But interpretation of urinary arsenic as a biomarker of arsenic exposure is difficult, because many factors are associated with arsenic metabolism (speciation) including age, sex, interindividual variability, capacity of

methylation, smoking status, absorption of arsenobetaine rich-products such as seafood, dietary intake, folate deficiency, ethnicity and chronic exposure (Marchiset-Ferlay et al., 2012).

Hair and nails: Arsenic concentration in hairs or nails has been used historically in forensic investigations to confirm acute poisoning, since high concentrations of arsenic can be detected at the base of the nail or hair up to 10–14 days following death (Slotnick and Nriagu, 2006). Human hair and nails arsenic concentrations have been reported to provide integrated measures of arsenic exposure (Agahian et al., 1990; Ali et al., 2010; Karagas et al., 1996). Previously Ali et al. (2010) and Karim et al. (2010) showed the strong positive association with drinking water, hair and nail arsenic levels indicating that hair and nail might be reliable biomarkers for chronic arsenic exposure. Sulfhydryl groups present in the keratin that comprises hair and nails, arsenic is therefore drawn to these parts of the body more exclusively. Since these tissues grow slowly, so they can be used as indicators of long-term exposure. Background arsenic levels in hair are < 1 µg/g (Hindmarsh, 2002) and in nails range from < 1.5 to 7.7 µg/g (Agahian et al., 1990; Hinwood et al., 2003).

Many epidemiological studies demonstrated strong relationship of arsenic concentration in drinking water with arsenic concentration in hair and nails (Ali et al., 2010; Cavar et al., 2005; Chiou et al., 1997; Hinwood et al., 2003; Karagas et al., 2000; Mandal et al., 2004). Arsenic in human nails has been often associated with concentrations of arsenic in drinking water, air, soil, dust and food (Slotnick and Nriagu, 2006).

Hair concentrations represent immediate exposure, as one centimeter of hair reflects approximately one month of exposure. On the other hand, nails capture historical exposure from several months to a year prior to sampling (Michaud et al., 2004).

Therefore, blood and urine arsenic concentration have been considered to reflect only recent and acute exposures, whereas arsenic concentrations in hair and nails reflect long-term exposure (Marchiset-Ferlay et al., 2012; Polissar et al., 1990; Pomroy et al., 1980).

1.12 Health effects of arsenic exposure

Long-term exposure to arsenic is a major threat to the public health. The characteristic health effects that result from ingestion of arsenic-contaminated drinking water are manifested slowly, and the diagnosis is usually straightforward. Several studies have clearly indicated that the toxicity of arsenic depends on the exposure dose, frequency and duration, age, sex, as well as on individual susceptibilities, genetic and nutritional factors (Ahsan et al., 2007; Maharjan et al., 2007; Milton et al., 2004; WHO, 2000). Chronic exposure to arsenic in drinking water is causally related to increased risks of cancer in the skin, lung, bladder, kidney, liver, colon and prostate (Chen et al., 2003a, 2003b; Smith et al., 1998). Many epidemiological studies have also shown that arsenic exposure is associated with a number of non-neoplastic diseases including cardiac disease, cerebrovascular disease, pulmonary disease, peripheral neuropathy, diabetes mellitus and different diseases of the arteries, arterioles, and capillaries (Engel and Smith, 2004; Lee et al., 2002; Rahman et al., 1999; Tseng et al., 2003; Hossain et al., 2012). In fact, arsenic affects almost all vital organs and systems of human body causing the damage or dysfunction (Hossain et al., 2012; Karim et al., 2010; Islam et al., 2011; Mandal et al., 2002). Adverse health effects of arsenic toxicity are manifested by increasing or decreasing levels of several soluble molecules and enzymes in human blood (Karim et al., 2010).

1.12.1 Dermatological effects

Skin lesions are a classical sign of chronic arsenic poisoning. Chronic exposure to arsenic by either ingestion or inhalation produce a variety of skin symptoms including diffused and spotted melanosis, leucomelanosis, keratosis, hyperkeratosis, dorsum, Bowen's disease and cancer. Skin disorders are well documented in several epidemiological studies conducted in different parts of the world in which the population are exposed to arsenic through drinking water (Ahsan et al., 2006; Chakraborti et al., 2003; Khan et al., 2003; Mazumder et al., 1998; Rahman et al., 2005). Resently, Yoshida et al. (2004) reported dose-response relationship between arsenic levels in drinking water and risk of skin lesions. Melanosis and keratosis are found at the primary stage of arsenic-induced all dermatological manifestations, leuko-melanosis and hyperkeratosis in the second stage and ultimately may turn to skin cancer such as Bowen's disease, basal cell and squamous cell carcinoma (Khan et al., 2003; Milton et al., 2003, Yoshida et al., 2004). Hyperpigmentation may occur, particularly in body areas where the skin tends to be a little darker (Shannon and Strayer, 1989). Photograph of some skin lesions are given below.

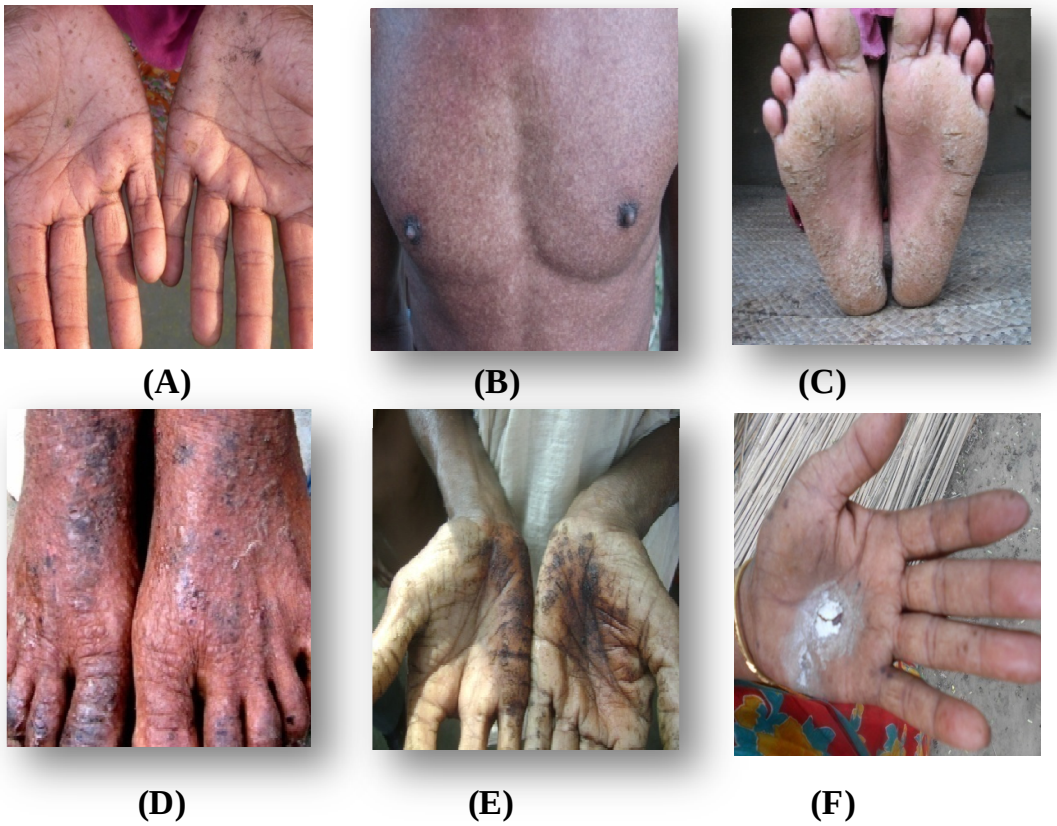


Figure 1.4 Photographs of arsenicosis.
Diffuse or spotted melanosis on palms (A) and on chest (B), blister on the soles (C), hyperkeratosis and ulceration on foots (D) and on palm (E), lesion on palm located with slaked lime (F).
[**Source:** Environmental Health Science Research group, Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi, Bangladesh]

1.12.2 Carcinogenic effects

The International Agency for Research on Cancer (IARC) has classified inorganic arsenic as Group 1 human carcinogen (IARC, 1987). Many different systems or organs within the body are affected by chronic exposure to inorganic arsenic, particularly because of its potential as a human carcinogen. A carcinogen is any substance that directly involved in causing cancer. Cancer is the leading cause of arsenic-related morbidity and mortality. The carcinogenic potential of arsenic was recognized over 110 years ago by Hutchison, who observed an unusual number of skin cancer in patients treated for various diseases with medical arsenicals (Klassen,

2008). Epidemiological studies have been carried out in different countries across the world which clearly demonstrated the causal relationship between environmental, occupational and medical exposure to inorganic arsenic and increased risks of cancer of the skin, lungs, urinary bladder, kidney, prostate, liver and other sites (Chen et al., 1992; IARC 2004; Smith et al., 1998; Wu et al., 1989; Yu et al., 2000). Ecological studies in Taiwan, Chile, Argentina and Australia and cohort studies from Taiwan and the USA demonstrated that long-term arsenic exposure increased the risks for kidney cancer (Chen et al., 1985, 1988a, 1992; Hopenhayn-Rich et al., 1998; Kurttio et al., 1999). Lung cancer is the leading cause of cancer-related mortality in the United States and also in rest of the world. An association between lung cancer and exposure to inorganic arsenic through different sources has been confirmed in several epidemiologic studies (Boyle and Maisonneuve, 1995; Hopenhayn-Rich et al., 1998). Liver is listed as a potential target organ for arsenic-induced carcinogenesis. Liver cancers can be developed from specific chronic liver diseases. Liver cirrhosis appears to be a primary cause of arsenic-related mortality in China, and is potentially associated with hepatocellular carcinoma (Liu et al., 1992, 2002). The association between environmental arsenic exposure and human liver cancers has been repeatedly reported (Centeno et al., 2002; Liaw et al., 2008).

1.12.3 Respiratory effects

Chronic exposure to inorganic arsenic naturally and occupationally is associated with a variety of respiratory problems including laryngitis, bronchitis, rhinitis, pharyngitis, shortness of breath, nasal congestion, hoarseness, chronic cough and perforation of the nasal septum (Dekundt et al., 1986; Mazumder et al., 2000; Milton and Rahman, 2002). The relationship between ingested arsenic and non-malignant respiratory

effects has been reported from Chile, India, and Bangladesh (Smith et al., 1998). A fatal case of arsenic trioxide inhalation manifested widespread as tracheobronchial mucosal and sub mucosal haemorrhages with mucosal sloughing, alveolar haemorrhages, and pulmonary edema (Gerhardsson et al., 1988). Chronic asthmatic bronchitis and asthma is a common complication of groundwater arsenic toxicity (Saha, 1995).

1.12.4 Hepatotoxic effects

Liver is the primary target organ for arsenic metabolism. Since the liver tends to accumulate arsenic with repeated exposures, hepatic involvement is reported most commonly as a complication of chronic arsenic exposure. Abnormal liver function manifested by gastrointestinal symptoms such as abdominal pain, indigestion, loss of appetite and by clinical elevations of serum enzymes, frequently occurs from exposure to arsenic (Mazumder, 2005; Liu et al., 1992). Islam et al. (2011) have demonstrated the dose-response relationship between arsenic exposure and serum hepatic enzymes used for liver function test in the individuals exposed to arsenic chronically in Bangladesh. Liver injury caused by chronic arsenic exposure is initially manifested by degenerative lesions with jaundice, progressing to non cirrhotic portal hypertension, fibrosis, cirrhosis and neoplasia such as hepacellular carcinoma (Centeno et al., 2002; Lu et al., 2001). Histological examination of the livers has revealed a consistent finding of portal tract fibrosis (Mazumder, 2005). The individuals who exposed more frequently to arsenic suffer from cirrhosis, which is considered to be a secondary effect of damage to the hepatic blood vessels. Hospitalized Indian arsenicosis patients have very high rates of hepatoportal sclerosis developed from drinking water contaminated with high level of arsenic (Dhawan et

al., 1983; Santra et al., 1999). Chronic arsenic exposure in animals can also produce liver endothelial cell damage, which subsequently damages parenchymal cells (Straub et al., 2007). All these studies clearly revealed that prolonged exposure to arsenic is associated with hepatomegaly, hepatic fibrosis and cirrhosis.

1.12.5 Renal effects

The kidneys are another important organ that accumulates inorganic arsenic with frequent exposure. Several epidemiological studies have shown the relationship between renal dysfunction and arsenic exposure through drinking water (Chen et al., 2011; Feng et al., 2013). The kidneys are the major route of arsenic excretion, as well as major site of conversion of pentavalent arsenic into the more toxic and less soluble trivalent arsenic (Tchounwou et al., 2003). Arsenic concentrates in the kidney during its urinary elimination affects the function of proximal convoluted tubules (Burton et al., 1995; Parrish et al., 1999). Acute renal dysfunction due to arsenic exposure is characterized by acute tubular necrosis and cast formation with increase in blood urea nitrogen and creatinine levels (Kimura et al., 2006). Several animal studies have reported renal effects following intermediate or chronic-duration of oral arsenic exposure (Brown et al., 1976, Karim et al., 2010, Rahman et al., 2012). The effects caused by arsenic include increased kidney weight, inflamed mitochondria and increased numbers of dense autophagic lysosome-like bodies in the proximal tubules, increased pigmentation in the proximal tubules, and cysts.

1.12.6 Neurological effects

Exposure to arsenic is associated with several form of neurological complications including impaired memory, poor concentration, Parkinson's disease, Guillain-Barre like neuropathy, verbal comprehension, encephalopathy, and peripheral neuropathy

(Bardullas et al., 2009; Piao et al., 2005; Vahidnia et al., 2006; Yip et al., 2002). The postulated mechanism for arsenic-induced neurotoxicity majorly involves oxidative stress with increased reactive oxygen species, lipid peroxides along with decrease in superoxide dismutase and reduced glutathione levels (Dwivedi and Flora, 2011). Arsenic exposure has been reported to alter metabolism of various neurotransmitters such as monoamines, acetylcholine, gamma amino butyric acid and glutamate (Rodriguez et al., 2002). The deficiency of thiamine is well known to induce neuronal complications. Arsenic exposure causes thiamine deficiency and inhibits pyruvate decarboxylase which elevates blood pyruvate and hence causes encephalopathy (Gopalkrisnan and Rao, 2006). Symptoms of chronic encephalopathy include persistent headache, diminished recent memory, distractibility, abnormal irritability, restless sleep, loss of libido, increased urinary urgency, and a bit increased effects of ethanol (Morton and Caron, 1989). Mental health problem (depression) is common neurological problem in the arsenic-endemic people in Bangladesh and China (Brinkel et al., 2009). A significant association between decreased reading and spelling performance and hair arsenic levels was found in a group of elementary school children suggesting that arsenic may also cause neurobehavioral effects (Moon et al., 1985).

1.12.7 Reproductive effects

Arsenic is a reproductive toxicant and a teratogen (Shalat et al., 1996). Arsenic and its methylated metabolites are reported to cross the placenta in both human and animals and thus arsenic is easily transferred to fetus at least in late stage of gestation (Concha et al., 1998; He et al., 2007; Vahter and Marafante, 1983). Several studies suggest that there is an association between arsenic exposure and adverse pregnancy outcomes,

such as spontaneous abortion, stillbirth, low birth weights, fetal loss and infant death (Ahmad et al., 2001; Milton et al., 2005; Rahman et al., 2007; von Ehrenstein et al., 2006). A significant decrease in sperm count and motility along with increase in abnormal sperm were observed at high concentration of arsenic exposure in mice (Pant et al., 2001). Arsenic exposure also affects weight and length during infancy and early childhood (Saha et al., 2012).

1.12.8 Genotoxic effects

Inorganic arsenic is generally recognized as a mutagenic agent. Several studies have been carried out exploring the genotoxic effect of inorganic arsenicals (Cohen et al., 2006; Yamanaka et al., 2004). Arsenic causes DNA damages, chromosomal abnormalities; epigenetic changes that alter DNA methylation status (Chanda et al., 2006; Kitchin, 2001; Rossman et al., 2004; Zhao et al., 1997). Chromosomal aberrations, DNA-protein cross-links and sister chromatid exchanges were observed in hamster embryo cells, human lymphocytes and fibroblasts after exposure to inorganic arsenic (Dong and Luo, 1993; Jha et al., 1992; Kochhar et al., 1996; Lee et al., 1985; Okui and Fujiwara, 1986; Rasmussen and Menzel, 1997; Wiencke and Yager, 1991). Arsenic-induced chromosomal aberrations are characterized by chromatid gaps, fragmentation, endoreduplication and chromosomal breaks. It has already been reported that both arsenic and its metabolites can have a variety of genotoxic effects, which may be mediated by oxidants or reactive oxygen species. All of these species also have effects on signaling pathways leading to proliferative responses. There are interesting differences in the activities of inorganic and organic species both in terms of target organ carcinogenicity, toxic and genotoxic mechanisms. Mass et al. (2001) indicated that exposure of human lymphocytes to

methylated trivalent arsenic causes direct DNA damage. A study using an earlier version of the alkaline elution method has indicated that arsenic induces DNA strand breaks in human fatal lung fibroblasts (Dong and Luo, 1993). Vuyyuri et al. (2006) reported that occupational exposure to arsenic among workers in a glass plant in India whose levels of blood arsenic were five times higher than in the control group had increased DNA damage in leukocytes. Li et al. (2001) reported that arsenic induced typical and various extents of DNA strand breaks in human cells via reactive oxygen species (ROS) in a dose-dependent manner. The most extensively studied DNA lesion is the formation of 8-hydroxyguanine (8-OH-G), one of the major products of DNA oxidation, which originates from the reaction of hydroxyl radical with guanine (Valko et al., 2006). 8-OH-G is a sensitive genotoxic marker of oxidative damaged DNA. Associations of arsenic exposure with increased urinary 8-OH-G concentrations have also been observed (Hu et al., 2006). Several studies showed that arsenic exposure causes epigenetic changes (Bailey and Fry, 2014; Reichard and Puga, 2010; Smeester et al., 2011; Hou et al.,2012). Epigenetic changes are the external modification of DNA without changing the sequences of bases. Hypo and hyper methylation of bases present in DNA are the main event in epigenetic changes. Epigenetic changes can be inherited to child from mother. Epigenetic alterations not only cause adverse effect on embryonic or neonatal growth but also can induce cancer or other deadly diseases in later life (Heindel, 2007; Vahter et al., 2008).

1.12.9 Cardiovascular effects

One of the major causes of chronic arsenic exposure-related morbidity and mortality is cardiovascular diseases (CVDs). CVDs are also leading causes of death globally. Atherosclerosis is the fundamental step for the development of CVDs. Proatherogenic

action of arsenic has been reported in several studies (Chen et al., 1988b; Chiou et al., 1997; Hossain et al., 2012; Karim et al., 2013; Rahman et al., 1999; Sohel et al., 2009; Tseng et al., 2003, 2005; Wang et al., 2002). Arsenic-induced CVDs in human may result from the interaction among genetic, environment and nutritional factors. Epidemiological studies have shown that arsenic ingestion through food or water may have serious effects on the human cardiovascular system including heart damage (myocardial depolarization, hypertrophy of the ventricular wall, cardiac arrhythmias), vascular damage (Raynaud's disease, blackfoot disease, arterial thickening), ischemic heart disease, cerebrovascular diseases, and hypertension (Chen et al., 1988b; Chiou et al., 1997; Hossain et al., 2012; Rahman et al., 1999; Sohel et al., 2009; Tseng et al., 2003, 2005; Wang et al., 2002). The first evidence of a link between CVDs and arsenic in drinking water came in 1980 from Antofagasta, Chile, with a report of 17 deaths from myocardial infarction in people under the age of 40 (Yuan et al., 2007). Increased risk of CVDs was reported in smelter workers due to arsenic exposure (Axelson et al., 1978; Lee-Feldstein 1989). It is believed that vascular endothelial cells play a pivotal role in arsenic-induced CVDs. Several epidemiological studies reported that chronic arsenic poisoning through ingestion of arsenic-contaminated water can affects the platelets which increase the risk of death in humans from various form of CVDs (Axelson et al., 1978; Lee et al., 2002; Lee-Feldstein, 1989; Wu et al., 1989). A dose-response relationship between prevalence of CVD and ingested arsenic was reported in north-eastern Taiwan (Chiou et al., 1997). Ischemia is localized tissue anaemia due to obstruction of the inflow of arterial blood. Mounting evidence indicated that arsenic increases mortality from ischemic heart disease (Chang et al., 2004; Chen et al., 2011; Tsai et al., 1999). Black foot disease a unique form of peripheral vascular disease, has been reported to be one of the important complication

of chronic arsenic toxicity in south-western Taiwan (Tseng, 1977). It is characterized by the severe systemic arteriosclerosis as well as dry gangrene and spontaneous amputations of affected extremities at end stages. Black foot disease has been mainly observed in Taiwan and it is possible that malnutrition contributes to its development. Increased prevalence of peripheral vascular disease has also been reported among residents with chronic arsenic exposure through drinking water in Taiwan, Chile, the USA, and Mexico (Chen et al., 2007; NRC, 1999, 2001; Tseng et al., 1996; Wang et al., 2007). There are several circulating markers that are implicated in the atherosclerotic event leading to CVDs. The blood-circulating molecules including lipoproteins, inflammatory and adhesion molecules have been reported to be involved in the formation of atherosclerotic lesions and many of them are predictive for atherosclerosis and CVD (Blankenberg et al., 2001; Hwang et al., 1997). Generally, lipid-related biomarkers such as TG, TC, LDL and HDL have been used as markers or surrogates for the risk of atherosclerosis (deGoma et al., 2008). Recently, oxidized form of LDL (Ox-LDL), rather than total LDL, has created a great attention for its role in the development of atherosclerosis (Heinecke, 1998; Steinberg, 1997). The oxidation of LDL is shown to be involved in the initiation of atherosclerosis (Steinberg, 1997). The levels of Ox-LDL in plasma have been used as a potential marker for oxidative stress in vascular systems (Heinecke, 1998; Steinberg, 1997). On the other hand, many studies have shown that the levels of HDL are inversely associated with the risk of atherosclerosis (Mertens and Holvoet, 2001). The protective effects of HDL against CVD have been considered to be mediated by its ability to remove cholesterol from artery-wall foam cells via 'reverse cholesterol transport'. However, recent findings have suggested that multiple functions of HDL, including its anti-oxidant and anti-inflammatory activities, are also involved in anti-

atherogenic properties of HDL (Bandeali and Farmer, 2012). Thus, the blood levels of Ox-LDL and HDL may reflect the balance between oxidative or inflammatory stress and anti-oxidant or anti-inflammatory protections in vascular systems (Mertens and Holvoet, 2001). In the recent publications from our laboratory Karim et al. (2013) have shown that arsenic exposure is associated with increased plasma ox-LDL level with the concomitant reduction of HDL cholesterol level. In the same paper, Karim et al (2013) has also demonstrated that chronic arsenic exposure is also associated with inflammatory molecules c-reactive protein (CRP), adhesion molecules (inter cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)). Very recently, same group (Rahman et al., 2015) has also reported that chronic exposure to arsenic is positively associated with proangiogenic molecule, vascular endothelial growth factor (VEGF). VEGF has also been reported to be associated with atherosclerotic plaque instability and rapture. Growing evidence has suggested uric acid as a putative marker of cardiovascular risk (Bos et al., 2006; Erdogan et al., 2005; Fukui et al., 2008; Ioachimescu et al., 2008; Mutluay et al., 2012; Niskanen et al., 2004; Pacifico et al., 2009; Puig et al., 1999; Storhaug et al., 2013; Zhang et al., 2012). High level of blood uric acid is an indicator of gout. Gout is a medical condition characterized by red, tender, hot and swollen joints caused by recurrent attacks of acute inflammation. Recent epidemiological data have found the association between CVDs and gout (Abbott et al, 1988; Krishnan et al, 2008). However, the association between arsenic exposure and blood uric acid is yet to be clarified.

1.13 Biochemistry and Metabolism of Uric Acid

Uric acid is a carbon, nitrogen, oxygen, and hydrogen containing heterocyclic compound (Figure 1.5) with the formula $C_5H_4N_4O_3$ (7, 9-dihydro-1H-purine-2, 6, 8(3H)-trione) and a molecular weight of 168 Daltons.

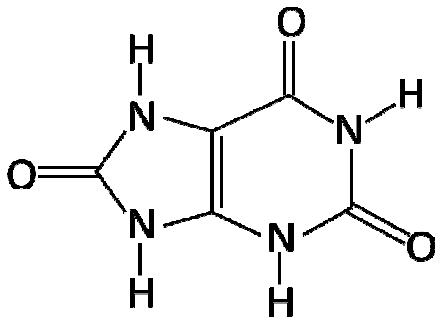


Figure 1.5 Structure of uric acid

Uric acid is the end product of purine metabolism in humans. Eating large amounts of foods high in purines raises uric acid levels in blood. The endogenous production of uric acid occurs in the liver, intestine, muscles, kidneys and the vascular endothelium. Production and excretion of uric acid are occurred through a complex process. Normally, most of the uric acid excreted from the human body through kidneys, while small amount is excreted through gut. Many enzymes are involved in the conversion of two purine bases in nucleic acid, adenine and guanine to uric acid. The final reactions of uric acid production are the conversion of hypoxanthine to xanthine and then to uric acid by the enzyme xanthine oxidase. Humans cannot oxidize uric acid to the more soluble compound allantoine due to the lack of the enzyme uricase, as is different from the other mammals. Because of the functional mutations during the early stage of hominoid evolution, humans and other primates have no functional uricase, which leads to higher blood uric acid levels when compared to rodents. The plasma uric acid (PUA) levels are varied by multiple factors including environmental

and genetic factors (Nath et al., 2007). Uric acid exists majorly as urate which is the salt of uric acid. Increase of urate concentration in blood ultimately leads to the increase formation of uric acid crystal. The normal or reference level of uric acid in blood is 2.4 to 5.7 mg/dL in women and 3.4 to 7.0 mg/dL in men. The solubility of uric acid in water is low, and in humans, the average concentration of uric acid in blood is close to the solubility limit (6.8 mg/dL). When the level of uric acid is higher than 6.8 mg/dL, crystals of uric acid form as monosodium urate. Studies have found that monosodium urate causes the inflammation observed in gout (Jin et al., 2012; Culleton et al., 1999; Wen et al., 2010; Popa-Nita et al., 2010) and may also contribute to the pathogenesis of vascular diseases (Jin et al., 2012; Abbott et al, 1988; Krishnan et al, 2008).

1.14 Dissertation aim

Arsenic is a potent environmental pollutant that is ubiquitously present in food, soil, water and airborne particles. The International Agency for Research on Cancer (IARC) classifies arsenic as a Group 1 carcinogen based on evidence that inorganic arsenic causes a variety of cancer in humans. People are generally exposed to arsenic through contaminated drinking water, food, and air-dust. Occupational exposure to arsenic may also occur through the inhalation of arsenic dusts in the production and distribution processes. However, contaminated drinking water has been recognized as the major source of human exposure to arsenic (Ali et al., 2010; Smith et al., 2000). Arsenic poisoning is a global problem since arsenic contamination of ground water has been discovered in many countries including Bangladesh, India, Pakistan, Argentina, Mexico, Chile, United State of America, Taiwan and China. Arsenic poisoning has taken into a serious turn affecting millions of people in Bangladesh

(Smith et al., 2000). Elevated levels of arsenic have been reported in 61 out of 64 districts (administrative blocks) in the country and the scale of disaster has exceeded the Chernobyl catastrophe in Ukraine and Bhopal accident in India (Smith et al., 2000). Many people have died of chronic diseases caused by prolonged exposure to arsenic. It has been assumed that 80-100 million people are at risk of arsenic poisoning in the country (Caldwell et al., 2003; Chowdhury, 2004; Chowdhury et al., 2000). Recent studies suggest that arsenic has entered the food chain in Bangladesh. Arsenic in food chain indicates that human exposure to arsenic is unavoidable. Ingestion of inorganic arsenic have been documented to be associated with a variety of diseases including cancers, cardiovascular diseases (CVDs), dermatitis, neurotoxicity, diabetes mellitus, renal failure and liver dysfunction (Tapio and Grosche, 2006; Karim et al., 2013; Wang et al., 2002; Guha Mazumder et al., 1998; Vahidnia et al., 2008; Meliker et al., 2007; Islam et al., 2011).

The elevated level of blood uric acid is associated with gout. Pathologically, the increased levels of PUA lead to the formation of crystal deposits in joints, tendons and other tissues. Besides the role of uric acid in the development of pathologic gout, however, a growing body of evidence has suggested that hyperuricemia is associated with the risk of CVDs including hypertension, metabolic syndrome, coronary artery disease, vascular dementia, stroke, preeclampsia, and kidney diseases (Cannon et al., 1966; Ford et al., 2007). Niskanen et al. (2004) conducted a prospective cohort study and showed that hypeuricemia is an independent risk factor for CVDs in middle-aged men. Furthermore, Storhaug et al. (2013) stated that serum uric acid is an independent marker of ischemic stroke in men, and the all-cause mortality in general Caucasian populations.

Many studies conducted in the arsenic-endemic populations in the world have clearly suggested that arsenic exposure is associated with CVDs (Chen et al., 2011; Karim et al., 2013; Wang et al., 2002). CVDs are the major causes of arsenic-related morbidity and mortality (Chen et al., 2011). Previously we and other groups have showed that arsenic exposure is associated with hypertension, a common form of CVDs (Hossain et al., 2012; Rahman et al., 1999). Although PUA is a putative marker for CVDs, the association between environmental arsenic exposure and PUA levels has not yet been documented. Therefore, the present study was designed to investigate the relationship of chronic human exposure to arsenic with PUA levels especially in connection with hypertension and circulating markers for CVDs.

Chapter 2

Materials and Methods

Materials and Methods

2.1 Chemicals and equipments for this study

2.1.1 List of chemicals and test kits

The important chemicals and test kits used in this study are mentioned below with their manufactures:

SL No.	Chemicals/kits	Company
1.	Uric Acid liquicolor	Human Diagnostic, Germany
2.	Nitric acid	MERCK, Germany
3.	Sulfuric acid	MERCK, Germany
4.	Ethanol	MERCK, Germany
5.	River water was used as a certified reference material. (NMIJ CRM 7202-a No.347).	National Institute of Advanced Industrial Science and Technology, Japan
6	Human hair as a certified reference material (CRM) (GBW09101)	Shanghai Institute of Nuclear Research Academia Sinica, China
7.	Urea liquicolor	Human Diagnostic, Germany
8.	HBsAg ELISA test kit	Medivent Diagnostic & Co. Ltd., Ireland

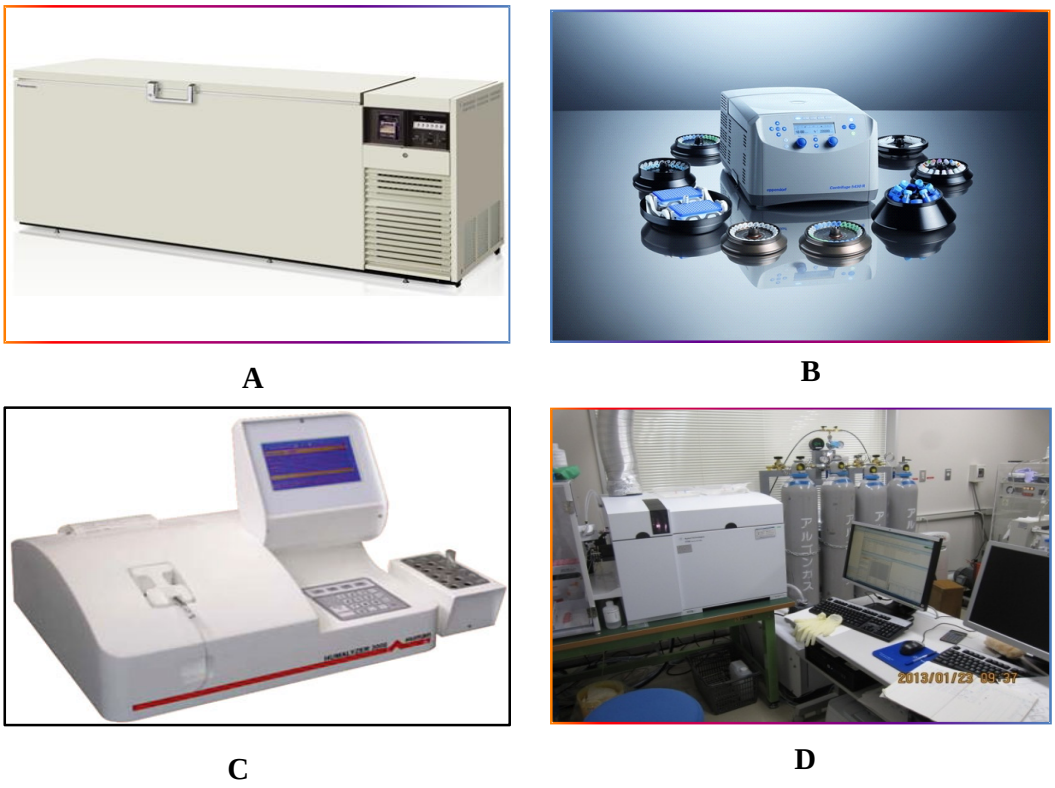
2.1.2 List of equipments

The following equipments were used in the study

SL No.	
1.	Inductively coupled plasma mass spectroscopy (ICP-MS) [Model No. HP-4500, Agilent Technologies, Kanagawa, Japan]
2.	EDTA-containing blood collection tubes
3.	Disposable syringes
4.	Ceramic blade cutter
5.	Sterile forceps
6.	Polypropylene bottles
7.	Eppendorf tubes
8.	Micropipettes
9.	Micropipette tips
10.	Centrifuge machine (Eppendorf, Model-5415 C)
11.	Temperature controlled water bath (Digisystem, Model: DSB-1000E)
12.	Incubator
13.	Pipettes
14.	Beakers
15.	Volumetric flasks
16.	Measuring cylinders
17.	Test tubes
18.	Distilled water bottles
19.	Conical flasks
20.	Sphygmomanometer

21. Digital electric balance
22. Autoclave (ALP Co. Ltd. KT-30L, Tokyo)
23. Refrigerator (4°C)
24. -80°C freezer (Model: CVK-UB, SANYO Electric Co. Ltd., Japan)
25. Analyzer (Humalyzer 3000, USA)
26. Multichannel micropipettes.
27. Microplate reader .

Some photographs of laboratory instruments used in this study:



A **B**

C **D**

Figure 2.1 Laboratory equipments used for this study
Ultra low temperature (-80°C) freezer (**A**) Centrifuge machine (**B**) Bio-analyzer (**C**) ICP-MS (**D**).

2.2 Study areas and subjects

Arsenic-endemic areas were selected from the North-West region of Bangladesh that included Marua in Jessore, Dutpatila, Jajri, Vultie and Kestopur in Chuadanga, and Bheramara in Kushtia district of Bangladesh and Chowkoli, a village in Naogaon

district with no history of arsenic contamination was selected as a non-endemic area. All sorts of confidentialities and rights of the study subjects were strictly maintained. Arsenic-endemic and non-endemic study areas for this study were selected as described previously (Ali et al., 2010; Hossain et al., 2012; Islam et al., 2011; Karim et al., 2010).

During the sample collection process, we were blinded to arsenic levels in the drinking water, hair and nails of the study participants. Attempt was made to match, as much as possible the following: age, sex and socioeconomic parameters (occupation, monthly income and education) of arsenic-endemic and non-endemic study subjects. The ratio of endemic to non-endemic subjects was approximately 2:1, and the ratio of male to female was approximately 1:1.

2.2.1 Inclusion criteria of the study subjects

Local residents (15-60 years of ages) who had lived for at least five years in arsenic-endemic and non-endemic areas were recruited for this study. The local residents who voluntarily participated in the study and gave their written consent were included in this study as study subjects.

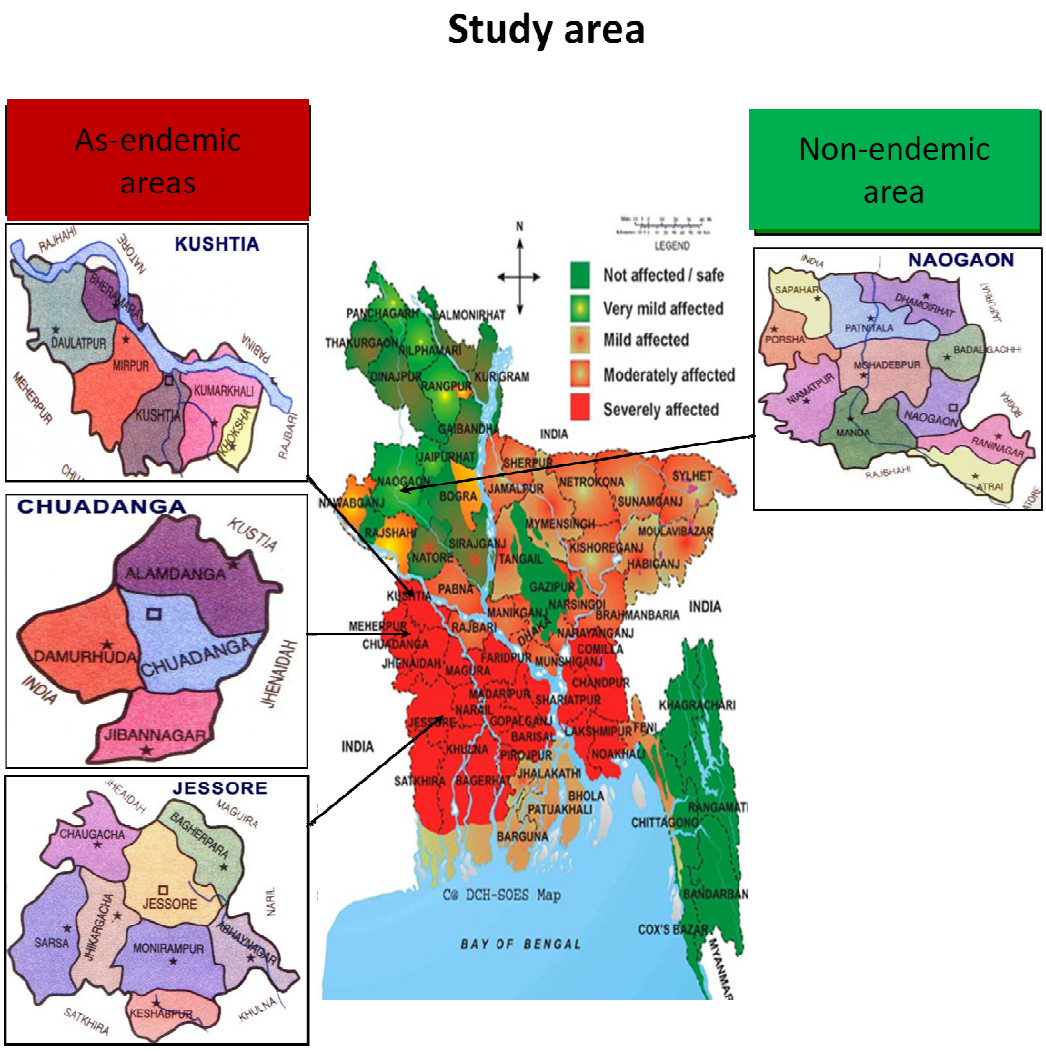


Figure 2.2 Study areas
(Source: School of Environmental Studies (SOES), Jadavpur University, India and Dhaka Community Hospital, Bangladesh. Available at: http://www.dchtrust.org/ars)

2.2.2 Excluding criteria of the study subjects

Pregnant and lactating mothers and the individuals who had a history of surgical operation, drug addiction, hepatitis B positive, hepatotoxic and anti-hypertensive drugs, malaria, kalazar, chronic alcoholism, history of hepatic, renal or severe cardiac diseases, and gout have been excluded from this study. Hepatitis B-positive study subjects were excluded based on the laboratory test.

2.2.3 Recruited study subjects

Of the 331 individuals who were approached, 9 were excluded according to the exclusion criteria [i.e., study candidates (n = 4) who had lived in arsenic-endemic areas for less than 5 years, pregnant and lactating mothers (n = 3) and had hepatic diseases (n = 2)]; thus a total 322 were finally recruited in arsenic-endemic areas. In non-endemic area 4 [i.e., study candidates (n = 2) who had lived in the non-endemic area for less than 5 years, pregnant mother (n = 1), study subjects who underwent recent surgical operation (n = 1)] from 165 were excluded. The final participants in non-endemic area were 161.

2.3 Ethical permission

Ethical permission was taken from the Institute of Biological Sciences, University of Rajshahi, Bangladesh (21/320-IAMEBBC/IBSc).

2.4 Questionnaire interview

Household visits were carried out in order to interview residents. Personal interviews of the study subjects were conducted by me and our research team. Personal interviews were conducted using a standard questionnaire (Annexure-1). Information obtained from the interview included the sources of water for drinking and daily household uses; water consumption history; socioeconomic status; occupation; food and eating habits; cigarette smoking; alcohol intake; personal and family history; history of diseases; physiological complications; major diseases; previous physicians' reports, Body Mass Index (BMI). Figure 2.3 shows some representative photographs of the field activities.

Figure 2.3 Photographs of the field activities
(A) Examination of a study subject **(B)** Personal interview of the study subject **(C)** Preparation for blood collection **(D)** Measurement of Body Mass Index (BMI) **(E)** Collection of nail and hair specimens **(F)** Collection of blood.

2.5 Collection of drinking water of the study subjects and analysis of arsenic

The drinking-water was entirely supplied by tube wells which were set up by the government or by individuals. The tube well water was used for drinking, cooking and other household purposes. Water samples from these tube wells were collected in acid-washed containers after the well was pumped for five minutes, as previously described (Van Green et al., 2008). Total arsenic concentrations in water samples were determined by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, HP-4500, Agilent Technologies, Kanagawa, Japan) after the addition of a solution of yttrium (10 ppb in 1.0% nitric acid) to all water samples as an internal standard for

ICP-MS analysis. The ion signals for arsenic and yttrium were monitored at m/z values of 75 and 79 respectively. All samples were determined in triplicate and the average values were used for the data analysis. The detection limit of ^{75}As was 0.03 µg/L. River water (NMIJ CRM 7202-a No.347 National Institute of Advanced Industrial Science and Technology, Japan) was used as a certified reference material (CRM). The average value (mean ± SD) of arsenic in the river water determined in triplicate by ICP-MS analysis was 1.06 ± 0.04 µg/L (reference value 1.18 µg/L).

2.6 Collection of hair and nail samples and analysis of arsenic

Arsenic levels in the hair and nails have been reported to provide integrated measures of arsenic exposure (Ali et al., 2010; Agahian et al., 1990; Karagas et al., 1996). Therefore, for the present study, we assessed hair and nail arsenic concentrations as exposure metrics, together with our assessment of the water arsenic concentrations. Nail samples were collected from each study subject as described previously (Schmitt et al., 2005). Hair samples (length approximately 1 cm) were collected from a region of the head close to the scalp behind the ear by using a ceramic blade cutter, and the samples were kept in polypropylene bottles (Paakkanen et al., 1998). Hair and nail samples were cleaned according to the method described by Chen and colleagues (Chen et al., 1999). Samples were immersed in 1% Triton X- 100, sonicated for 20 minutes and then washed five times with milli-Q water. The washed samples were allowed to dry at 60°C overnight in a drying oven. The hair and nail samples were then digested with concentrated nitric acid using a hot plate at 70°C for 15 minutes and at 115°C for 15 minutes. After cooling, the samples were diluted with 1.0% nitric acid containing yttrium (10 ppb) and concentrations of ^{75}As and ^{79}Y in these samples were determined by ICP-MS (HP-4500, Agilent Technologies, Kanagawa, Japan). All

samples were determined in triplicate and the average values were used for the data analysis. The accuracy of arsenic measurement was verified using a CRM "cod fish powder" (NMIJ CRM 7402-a, National Institute of Advanced Industrial Science and Technology, Japan). The average value (mean ± SD) of arsenic in the cod fish powder, 34.9 ± 2.35 μg/g (reference value 36.7 μg/g), was determined in triplicate by the above-mentioned digestion method, followed by ICP-MS analysis.

2.7 Collection of blood plasma

All study subjects were asked to convene at a designated location nearby. All study subjects were requested to fast overnight (10-12h) to collect fasting blood samples. Blood samples (5-7 ml) were collected from each individual by venipuncture into the EDTA-containing blood collection tubes (plain tubes). Whole blood was then placed immediately on ice and subsequently centrifuged at 1,600 x g for 15 min at 4^{o}C. Plasma supernatant was then taken. Finally serum and plasma samples were stored at -80^{o}C freezer.

2.8 Blood pressure measurement

The standard protocol for measuring blood pressure recommended by World Health Organization (WHO) was used in this study. After the study subjects had rested for 20 min or longer, both systolic and diastolic blood pressures (SBP and DBP) were measured three times with a mercury sphygmomanometer with subjects sitting. SBP and DBP were defined at the first and fifth phase Korotkoff sounds respectively. The average of three measurements was used for the analysis. Hypertension was defined as a SBP of ≥140 mmHg and a DBP of ≥ 90 mmHg on three repeated measurements (James et al., 2014).

2.9 Screening of hepatitis B

All blood samples were checked for hepatitis B using a 3rd-generation HBsAg ELISA test kit (Medivent Diagnostic & Co. Ltd., Ireland) according to the manufacturer's protocol.

2.10 Measurement of PUA

PUA was measured using an assay kit (Human Diagnostic, Germany) through analyzer (Humalyzer 3000, USA) at 520 nm according to the manufacture's protocol. All plasma samples were analyzed in duplicate and the mean values were used.

Principle of methods:

Uric acid is converted into allantoin and H_2O_2 by reaction with uricase enzyme. The formed H_2O_2 reacts under catalysis of peroxides with 3, 5-dichlro-2-hydroxybenzenesulfonic acid (DCHBS) and 4-aminophenazone (PAP) to give a red-violet quinoneimine dye as indicator. The color formed is proportional to plasma uric acid concentration in the sample which has been measured through analyzer at 520 nm.

Reaction principle:

$$\text{Uric acid} + O_2 + 2H_2O \xrightarrow{\text{Uricase}} \text{allantoin} + CO_2 + H_2O_2$$

$$2H_2O_2 + \text{DCHBS} + \text{PAP} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + HCl + 4H_2O$$

Calculation:

To obtain uric acid concentration from plasma following formula was used according to the protocol.

$$C = 8 \times \frac{\Delta A \text{ Sample}}{\Delta A} \text{ ng/dl}$$

All blood samples were analyzed in duplicate and then mean values were taken.

2.11 Measurement of BUN

BUN from the collected plasma specimens was measured using commercially available assay kit manufactured by Human Diagnostic, Germany through analyzer at 570-600 nm according to the manufacture's protocol.

Reaction Principle:

Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. In a modified Berthelot reaction, the ammonium ions react with hypochlorite and salicylate to from a green dye. The absorbance increases at 580 nm is proportional to the blood urea nitrogen concentration in the sample.

Type of specimen: Plasma

Reagent Composition:

Contents	Concentration
Reagent 1 (RGT1)	
Phosphate buffer (pH 7.0)	120 mmol/l
Sodium salicylate	60 mmol/l
Sodium nitroprusside	5 mmol/l
EDTA	1 mmol/l
Reagent 2 (RGT2)	
Phosphate buffer (pH < 13)	120 mmol/l
Hypochlorite	0.6 g/l Cl
Enzyme	
Urease	> 500KU/l
Standard	
Urea	13.3 mmol/l
Equivalent to BUN	6.2 mmol/l
Sodium azide	0.095 %

Reagent Preparation:

RGT2 and STD were ready for use. The enzyme reagent 1a was prepared by mixing the contents of bottle ENZ with bottle RGT1 as a ratio 1:100.

Assay:

Wave length	570-600 nm
Optical path	1 cm
Temperature	37°C
Measurement	Against reagent blank

Pipetting Scheme:

Pipette into cuvettes	Reagent blank	Sample or STD
Sample/STD		10 µl
Enzyme reagent 1a	1000 µl	1000 µl
Mix, incubate for 3 min at 37º C.		
RGT2	1000 µl	1000 µl
Sample was mixed with reagents and incubates for 5 min at 37º C. Absorbance of the sample was measured against reagent blank.		

Calculation:

To obtain urea concentration from plasma following formula was used according to the protocol:

$$\text{Conc.} = \frac{\text{(A) Sample}}{\text{(A) Standard}} \times 80\,(\text{factor}) = \text{mg/dL urea in the sample}$$

For, mmol/L factor = 13.3

All serum samples were analyzed in duplicate and then mean values were taken.

2.12 Statistical analysis

Statistical analysis of this study was performed using the Statistical Packages for Social Sciences (SPSS version 17.0, SPSS Inc., Chicago, IL) software. Characteristics and blood biochemistry data of the male and female study subjects from arsenic-endemic and non-endemic areas were analyzed by independent sample *t*-test and chi-square test. Normality of the distribution of variables was verified by a Q-Q plot. Because of skewed distributions of arsenic exposure metrics, log transformed values were used for statistical analysis. Correlations of drinking water arsenic concentrations with hair and nail arsenic concentrations were analyzed by Spearman Correlation coefficient test. Differences of PUA levels of the male and female study

subjects in arsenic-endemic and non-endemic areas were analyzed by Independent sample t-test. Correlations of PUA levels with arsenic concentrations in the drinking water, hair and nails were evaluated by Spearman correlation coefficient test. Subsequently, multiple regression analyses were performed to examine the associations of PUA levels with arsenic exposure metrics and other variables. The study subjects in the arsenic-endemic areas were stratified through frequency test into 'high' and 'medium' exposure group based on the concentrations of arsenic in the drinking water, hair, and nails. The study subjects in the non-endemic area were used as a reference group ('low' exposure group). PUA levels in the low, medium and high exposure groups were analyzed by one-way ANOVA followed by Bonferroni multiple comparison tests. Study subjects in arsenic-endemic and non-endemic areas were further divided into three ($\leq$ 10 µg/L, 10.1-50 µg/L and > 50 µg/L) groups based on the regulatory upper limit for water arsenic concentrations set by WHO (10 µg/L) and Bangladesh Government (50 µg/L). PUA levels in the three groups were evaluated by one-way ANOVA (Bonferroni test). Correlations of DBP and SBP with PUA were analyzed by Spearman correlation coefficient test. Multiple regression analyses were performed for the association of blood pressure (DBP and SBP) with arsenic exposure metrics, PUA levels, and other variables. Independent sample *t*-test was performed to compare the PUA levels between normotensive and hypertensive study subjects. Correlations of PUA with C-reactive protein (CRP), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were evaluated by Spearman correlation coefficient test. A value of $p < 0.05$ was considered statistically significant.

Chapter 3

Results

3.1 Descriptive characteristics of study subjects

Table 3.1 shows the general characteristics of the male and female study subjects in the arsenic-endemic and non-endemic areas. Of the 483 study subjects, 168 were males and 154 were females from arsenic-endemic areas, and 75 were males and 86 were females from non-endemic areas. The average ages of male study subjects in arsenic-endemic and non-endemic areas were 37.52 ± 12.92 and 36.44 ± 12.70 years, respectively, and those were 37.29 ± 10.88 and 36.47 ± 10.46 years in female study subjects, respectively. Arsenic concentrations in the drinking water, hair and nails of the study subjects in arsenic-endemic areas were approximately 70, 19 and 6 times higher in male and 72, 12 and 8 times higher in female groups respectively, than those of non-endemic areas. Because attempts were made to match the age, sex and socioeconomic parameters (occupation, education and monthly income) between arsenic-endemic and non-endemic study subjects, no significant differences were observed in those parameters between the two study groups. Most of the male study subjects in the both arsenic-endemic and non-endemic areas were farmers, whereas most of the female study subjects were housewives. A good number of study subjects (49.40% and 46.67% male study subjects in arsenic-endemic and non-endemic areas, respectively, and 55.19% and 62.79% female study subjects in arsenic-endemic and non-endemic areas, respectively) had no formal education. Percentages of higher (graduation) level education in both arsenic-endemic and non-endemic study subjects were very low. The average (Mean ± SD) monthly incomes of the study individuals were also low. All the socioeconomic parameters (occupation, education and monthly income) indicated that the study populations in arsenic-endemic and non-endemic areas were in low socioeconomic conditions. The DBP and SBP in arsenic-endemic

male and female groups were significantly ($p < 0.001$) higher than those of non-endemic subjects. Accordingly, the percentages of hypertensive subjects were also significantly ($p < 0.01$) higher in both male and female subjects in arsenic-endemic areas than those in non-endemic areas. No significant difference was found in the percentages of tobacco smokers between arsenic-endemic and non-endemic areas. No females were found to be a smoker. This was expected since Bangladeshi women generally do not smoke cigarettes. None of the study subjects admitted to drink alcohol because of the social and religious restriction on alcohol drinking. The averages (Mean ± SD) BMI of the male study subjects in arsenic-endemic and non-endemic areas were 20.03 ± 2.77, 20.57 ± 2.17 kg/m^2, respectively, whereas in the case of female study subjects, these were 21.34 ± 3.62 and 21.41 ± 3.20 kg/m^2, respectively. BUN levels of both male and female study subjects in arsenic-endemic and non-endemic areas were almost similar.

Table 3.1 Descriptive characteristics of the study subjects

Characters	Male			Female		
	Non-endemic	**Endemic**	***p*-value**	**Non-endemic**	**Endemic**	***p*-value**
Study subjects (n)	75	168		86	154	
Age[a]	36.44 ± 12.70	37.52 ± 12.92	0.542*	36.47 ± 10.46	37.29 ± 10.88	0.563*
Water As (µg/L)[b]	0.73 (6.79)	72.75 (6.20)	< 0.001*	0.54 (8.12)	68.74 (6.13)	< 0.001*
Hair As (µg/g)[b]	0.19 (2.19)	3.00 (2.69)	< 0.001*	0.31 (2.19)	2.84 (2.91)	< 0.001*
Nail As (µg/g)[b]	0.95 (2.32)	5.81 (2.41)	< 0.001*	0.84 (2.32)	6.04 (2.68)	< 0.001*
Occupation [n (%)]						
Male						
Farmer	61 (81.33)	139 (82.74)				
Student	6 (8.00)	8 (4.76)	0.483†			
Business	1 (1.33)	4 (2.38)				
Others	7 (9.33)	17 (10.12)				
Female						
House wives				74 (86.04)	133 (86.36)	
Worker				10 (11.63)	19 (12.34)	0.411†
Student				0	1 (0.65)	
Others				2 (2.33)	1 (0.65)	
Education [n, (%)]						
No formal education	35 (46.67)	83 (49.40)	0.635†	54 (62.79)	85 (55.19)	0.129†
Primary	31 (41.33)	58 (34.52)		29 (33.72)	54 (35.06)	
Secondary	6 (8.00)	21 (12.50)		3 (3.49)	13 (8.44)	
Graduate	3 (4.00)	6 (3.57)		0	2 (1.30)	
Income/month (US$)[a]	26.56 ± 10.19	25.53 ± 11.04	0.491*	22.70 ± 5.43	23.54 ± 9.52	0.382*
DBP (mm Hg)[a]	70.07 ± 8.60	76.46 ± 8.95	< 0.001*	69.42 ± 9.81	77.79 ±12.41	< 0.001*
SBP (mm Hg)[a]	110.13 ± 11.24	116.70 ± 13.74	< 0.001*	110.35 ± 15.83	121.75 ± 20.58	< 0.001*
Hypertension [n, (%)]						
Yes	0	18 (10.71)	< 0.01†	3 (3.50)	28 (18.18)	< 0.01†
No	75 (100)	150 (89.29)		83 (96.50)	126 (81.82)	
Smoking [n, (%)]						
Yes	27 (36.00)	63 (37.50)	0.823†	0	0	
No	48 (64.00)	105 (62.50)		86 (100)	154 (100)	
Alcohol Intake	-	-	-	-	-	-
BMI (kg/m^2)[a]	20.57 ± 2.17	20.03 ± 2.77	0.135*	21.41 ± 3.20	21.34 ± 3.62	0.874*
BUN (mg/dL)[a]	9.67 ± 2.75	9.40 ± 2.30	0.449*	8.34 ± 2.12	8.71 ± 2.51	0.223*

Abbreviations: As, Arsenic. [a] Mean ± SD; [b] Geometric Mean (SD). BMI was calculated as body weight (kg) divided by height squared (m^2). **p*- values were from independent sample *t*-test, and †*p*-values were from chi-square test. Others: included village doctor and rickshaw puller.

3.2 Correlations of drinking water arsenic with hair and nail arsenic concentrations in male study subjects

Drinking water arsenic concentrations of the male study subjects in arsenic endemic and non-endemic areas showed a strong positive association (r_s = 0.770, p < 0.001) with the arsenic concentrations in hair (Figure 3.1A). Similarly, a positive relationship (r_s = 0.756, p < 0.001) was observed between drinking water and nail arsenic concentrations (Figure 3.1B).

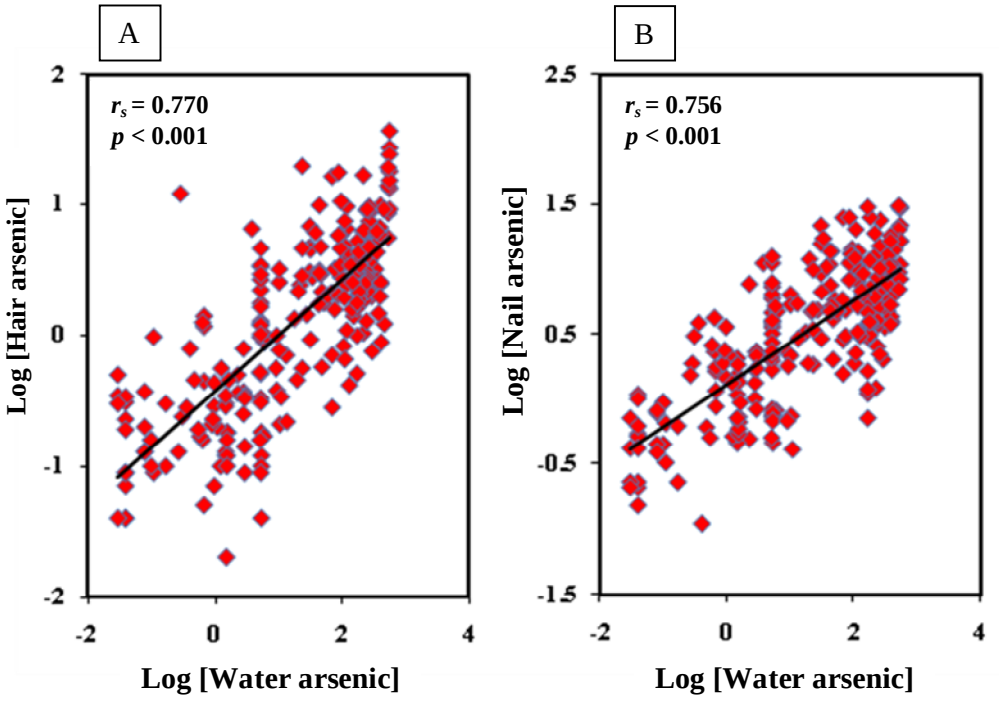


Figure 3.1. Correlations of drinking water arsenic with hair (A) and nail (B) arsenic concentrations of the male study subjects. Arsenic concentrations in the drinking water (µg/L), hair (µg/g) and nails (µg/g) of the study subjects were used after log transformation. r_s and p- values were from Spearman correlation coefficient test.

3.3. Correlations of drinking water arsenic with hair and nail arsenic concentrations in female study subjects

Like males, in the case of female study subjects in arsenic-endemic and non-endemic areas, positive and significant associations were also found between drinking water and hair arsenic (r_s = 0.767, p < 0.001), and between water and nail arsenic (r_s = 0.788, p < 0.001) concentrations (Figure 3.2A and 3.2B).

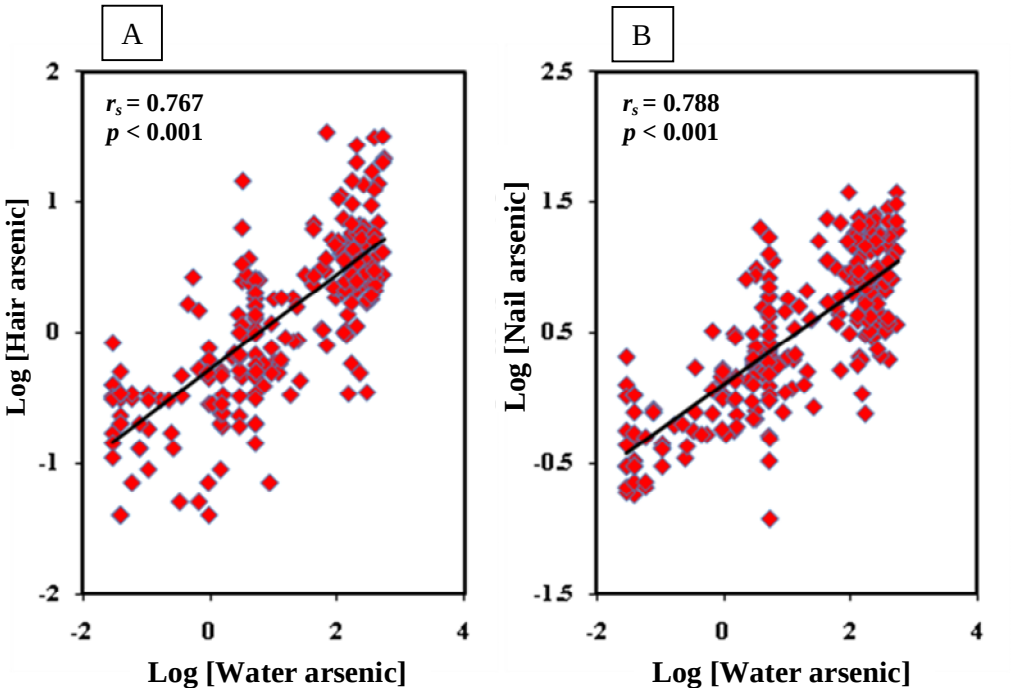


Figure 3.2 Correlations of drinking water arsenic with hair (A) and nail (B) arsenic concentrations of the female study subjects. Arsenic concentrations in the drinking water (µg/L), hair (µg/g) and nails (µg/g) were used after log transformation. r_s and p-values were from Spearman correlation coefficient test.

3.4 Comparisons of PUA levels between arsenic–endemic and non-endemic study subjects

Since the base line PUA levels in males and females are different, we compared the PUA levels between arsenic-endemic and non-endemic study populations separately in both sexes. The average PUA levels in both male and female groups in arsenic-endemic areas were significantly ($p < 0.001$) higher than those in non-endemic area (Figure 3.3A and 3.3B).

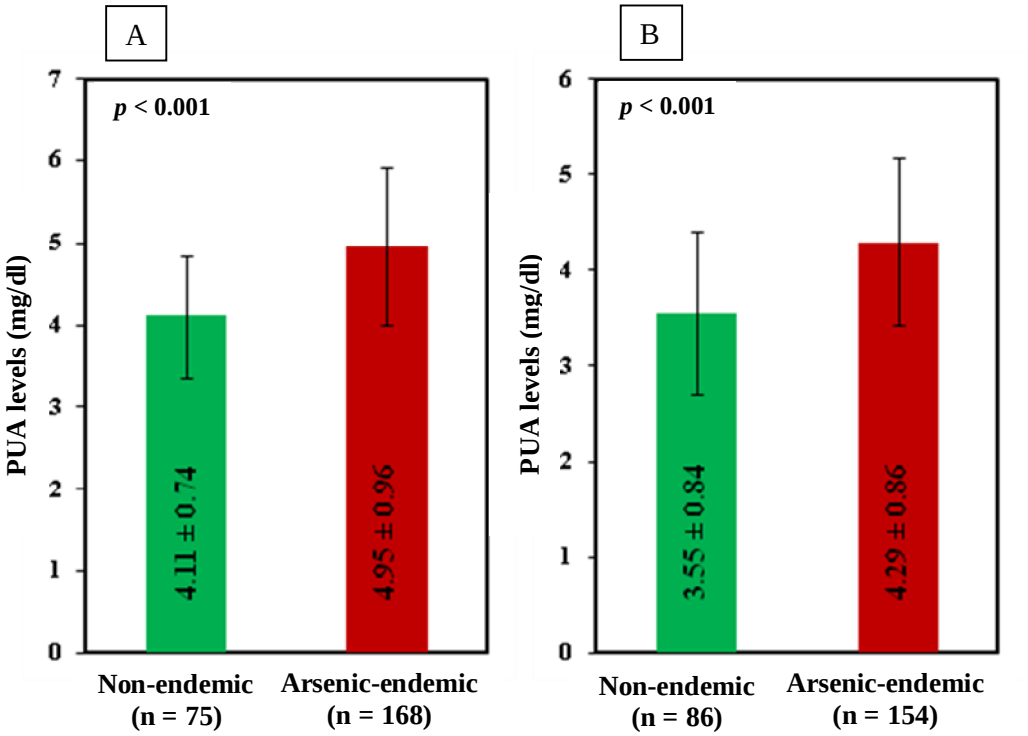


Figure 3.3 PUA levels in male (A) and female (B) study subjects in non-endemic and arsenic-endemic areas. *p*-values were from Independent sample t-test.

3.5 Correlation of arsenic exposure metrics (drinking water, hair and nail arsenic concentrations) with PUA levels in male study subjects

Figure 3.4 shows the correlations of PUA levels with arsenic concentrations in the drinking water, hair and nails of the male study population. A significant positive (r_s = 0.410, p < 0.001) correlation was observed between PUA levels and arsenic concentrations in the drinking water. Similar relationships were also observed between PUA and hair arsenic concentrations (r_s = 0.382, p < 0.001), and between PUA and nail arsenic concentrations (r_s = 0.339, p < 0.001).

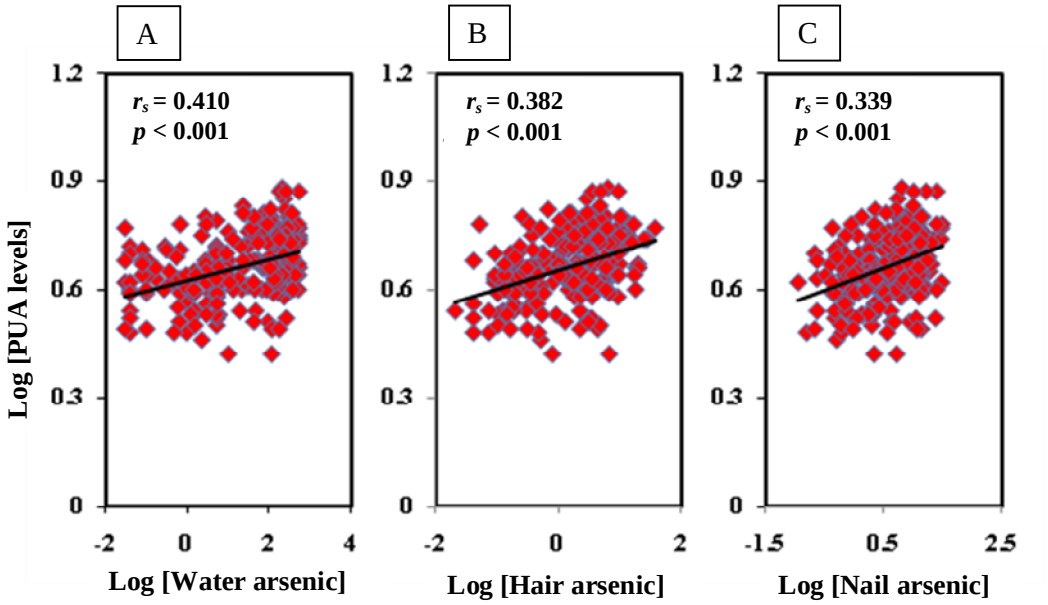


Figure 3.4 Correlations of PUA levels with drinking water (A), hair (B) and nail (C) arsenic concentrations of male study subjects. Log-transformed values of arsenic concentrations and PUA were used. r_s and p-values were from Spearman correlation co-efficient test.

3.6 Correlation of arsenic exposure metrics (drinking water, hair and nail arsenic concentrations) with PUA levels in female study subjects

Figure 3.5 shows the correlations of PUA levels with arsenic concentrations in the drinking water, hair and nails of the female study populations. PUA levels showed a significant positive (r_s = 0.324, p < 0.001) association with arsenic concentrations in the drinking water. Similar relationships were also observed between PUA and hair arsenic concentrations (r_s = 0.324, p < 0.001) and between PUA and nail arsenic concentrations (r_s = 0.205, p < 0.01).

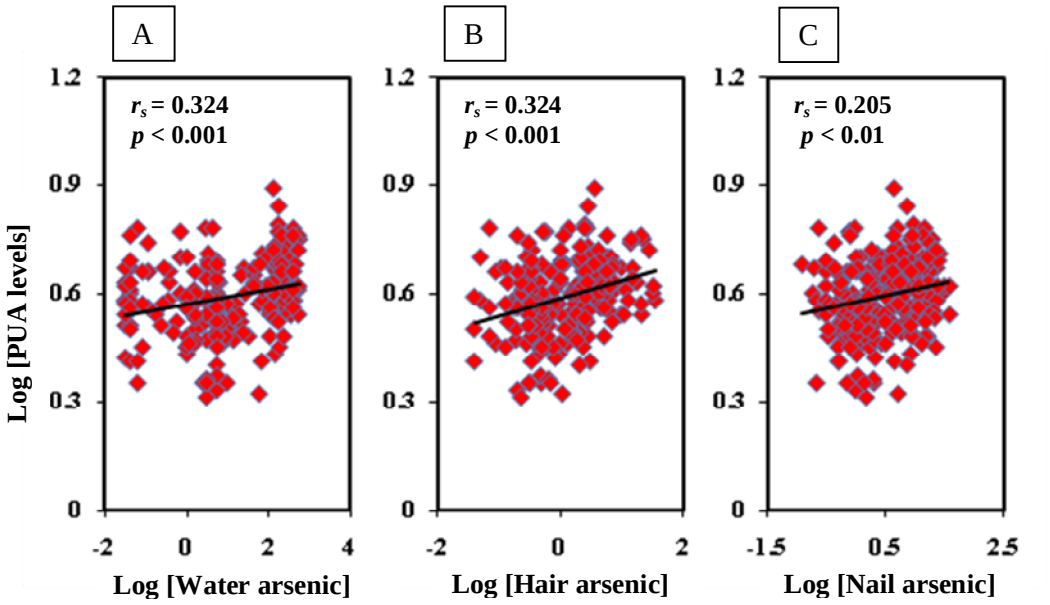


Figure 3.5 Correlation of PUA levels with drinking water (A), hair (B) and nail (C) arsenic concentrations in female study subjects. Log-transformed values of arsenic exposure metrics and PUA were used. r_s and p-values were from Spearman correlation co-efficient test.

3.7 Associations of arsenic exposure metrics with PUA levels through multiple regression analyses

We performed multiple regression analyses to examine the associations of arsenic exposure with PUA levels and other relevant variables (age, BMI, smoking habit and BUN). Water, hair, and nail arsenic concentrations showed significant and the highest β-coefficients among the independent variables in both males and females (Table 3.2). Age, BMI, smoking and BUN did not show significant β-coefficients in males. In females, BUN showed significant β-coefficients, but their values were lower than the water, hair and nail arsenic concentrations. These data suggested that arsenic exposure had the highest contributions on the elevation of PUA levels even after the adjustment of other confounding factors including BUN.

Table 3.2 Association of arsenic exposure metrics with PUA levels through multiple regression analyses

Independent variables	Dependent variable (PUA)				
	Males			Females	
	β-Coefficient	*p*-value		β-Coefficient	*p*-value
Water As	0.317	< 0.001	Water As	0.176	< 0.001
BMI	0.037	0.099	BUN	0.062	0.015
Age	-0.005	0.254	Age	-0.003	0.540
BUN	0.022	0.356	BMI	0.007	0.669
Smoking	0.036	0.764			
Hair As	0.599	< 0.001	Hair As	0.410	< 0.001
BMI	0.043	0.057	BUN	0.070	0.006
Age	-0.008	0.077	BMI	0.005	0.752
BUN	0.031	0.195	Age	-0.001	0.792
Smoking	0.029	0.811			
Nail As	0.686	< 0.001	Nail As	0.265	0.010
BMI	0.044	0.056	BUN	0.072	0.006
Age	-0.006	0.236	Age	-0.003	0.602
Smoking	0.074	0.545	BMI	0.008	0.632
BUN	0.020	0.421			

Log-transformed values of arsenic exposure metrics were used.

3.8 Dose-response relationship of water arsenic concentrations with PUA levels

Dose-response relationship is critically important for the assessment of risk and magnitude of the effect of a toxic substance. Therefore, next we investigated the dose-response relationship between water arsenic concentrations (as an external exposure metric) and PUA levels. Both male and female study subjects in arsenic-endemic areas were split into two arsenic exposure groups (medium and high) based on the drinking water arsenic concentrations, and non-endemic population was used as a low or reference group. Post-hoc Bonferroni multiple comparison test revealed that PUA levels were significantly different for medium versus low ($p < 0.001$), high versus low

($p < 0.001$), and high versus medium ($p < 0.05$) exposure groups in both male and female study subjects.

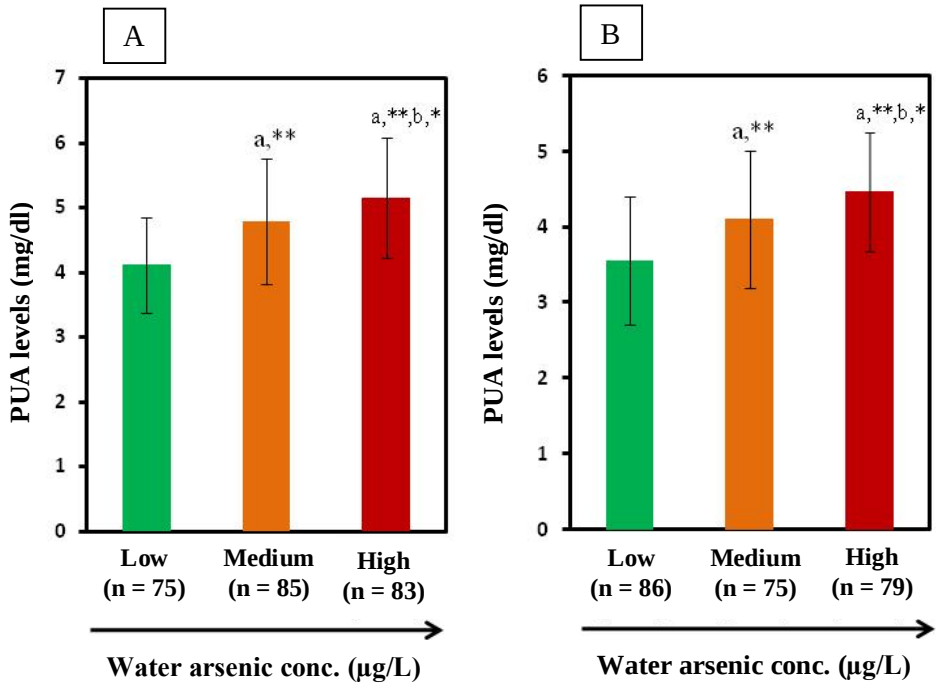


Figure 3.6 Dose-response relationship of PUA levels with water arsenic concentrations both in male (A) and female (B) study subjects.
[a]Significantly different from low group. [b]Significantly different from medium group. **, $p < 0.001$; *, $p < 0.05$. p-values were from one way ANOVA test.

3.9. Dose-response relationship of hair arsenic concentrations with PUA levels

Like water arsenic concentrations, both male and female study subjects in arsenic-endemic areas were split into two arsenic exposure groups (medium and high) based on the hair arsenic concentrations (as an internal exposure metric), and non-endemic population was used as a low or reference group. We found that PUA levels were higher in the medium and high exposure groups than the low exposure group in both males and females.

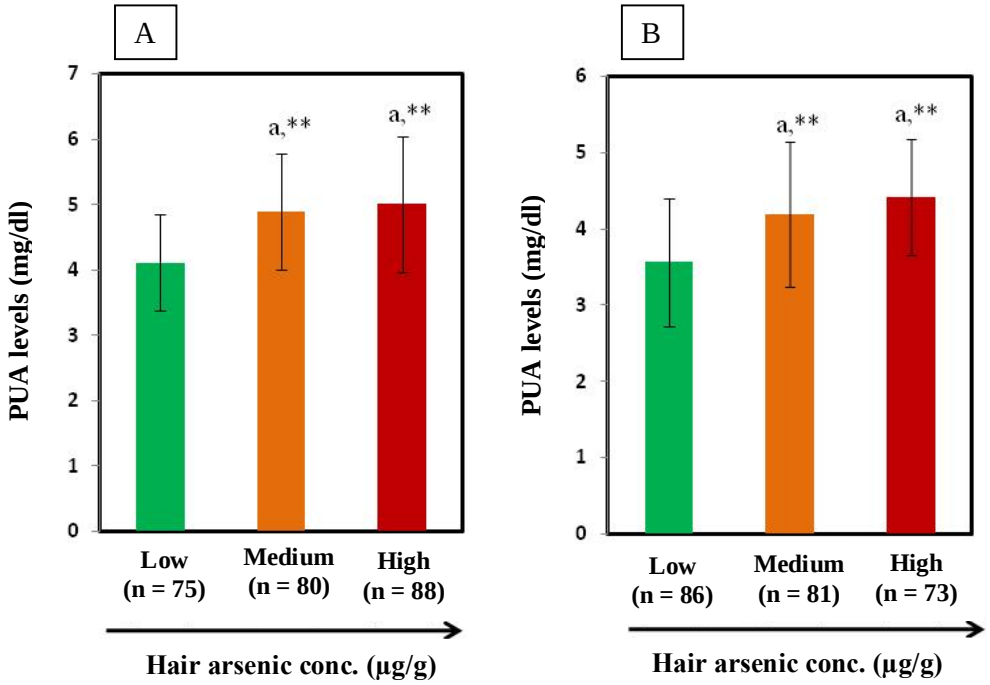


Figure 3.7 Dose-response relationship of PUA levels with hair arsenic concentrations both in male (A) and female (B) study subjects.

[a]Significantly different from low group. **, $p < 0.001$. *p*-values were from one way ANOVA test.

3.10 Dose-response relationship of PUA levels with nail arsenic concentrations

Finally, we evaluated the dose-response relationship between nail arsenic concentrations (another internal arsenic exposure metric) and PUA levels splitting the study subjects into three groups where non-endemic study subjects were considered as low or reference group. We found that PUA levels were higher in the medium and high exposure groups than the low exposure group in both males and females.

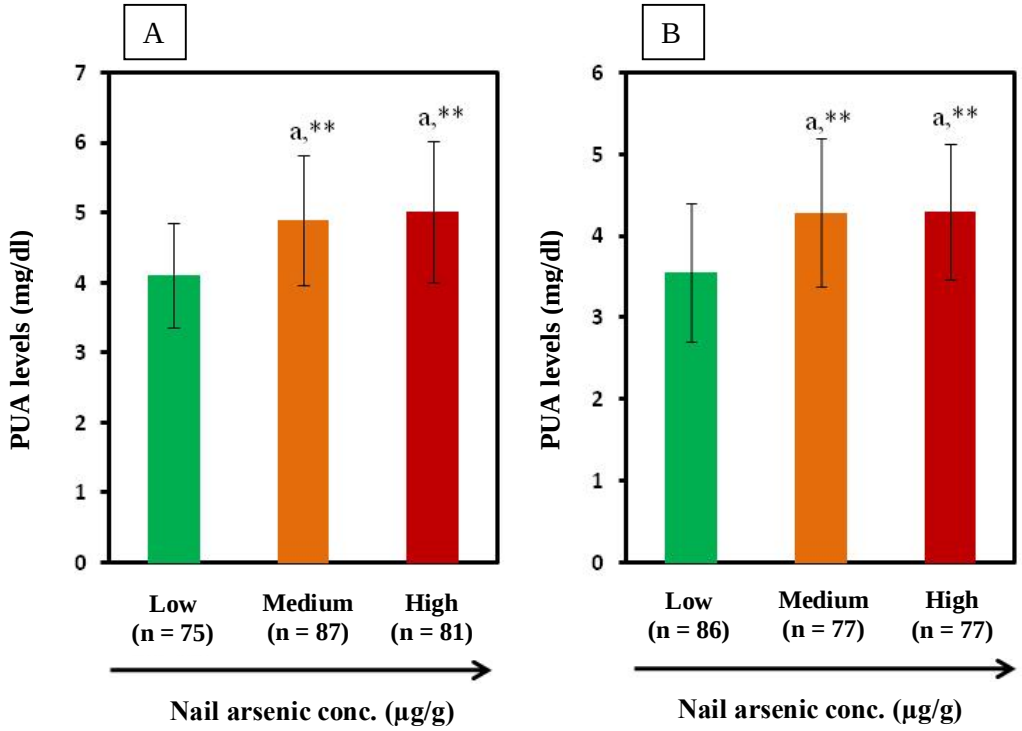


Figure 3.8 Dose-response relationship of PUA levels with nail arsenic concentrations both in male (A) and female (B) study subjects.
[a]Significantly different from low group. **, $p < 0.001$. *p*-values were from one way ANOVA test.

3.11 Comparisons of PUA levels in three groups based on the regulatory upper limits of water arsenic concentrations set by WHO and Bangladesh Government

Finally we divided the male and female study subjects into three groups (≤ 10 µg/L, 10.1-50 µg/L and > 50 µg/L) based on the regulatory upper limit of water arsenic concentrations set by WHO (10 µg/L) and Bangladesh Government (50 µg/L) in order to evaluate the dose-response relationship of water arsenic concentrations with PUA levels. We found that PUA levels were significantly higher in the > 50 µg/L groups in both male and female study subjects than the ≤ 10 µg/L group. Further the 10.1-50 µg/L groups showed significantly ($p < 0.001$) higher PUA levels than the ≤ 10 µg/L groups in male but not in female study subjects. PUA levels in the > 50 µg/L group of female study subjects was significantly ($p < 0.001$) higher than those of the 10.1-50 µg/L group.

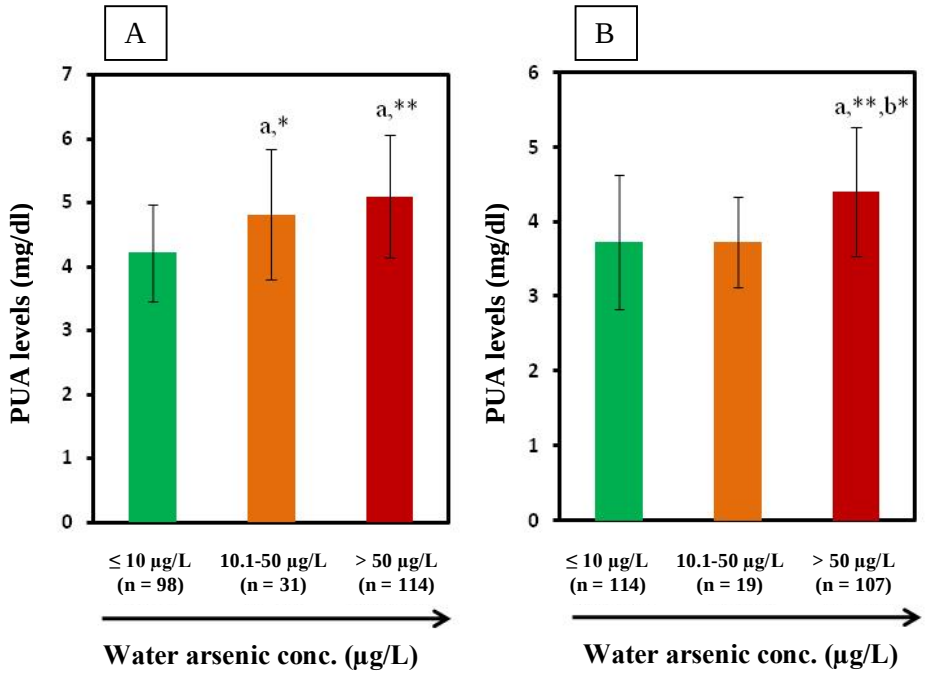


Figure 3.9 PUA levels in three groups based on upper limit of water arsenic concentrations set by WHO and Bangladesh Government both in male (A) and female (B) study subjects. [a]Significantly different from ≤ 10 µg/L group, [b]Significantly different from 10.1 - 50 µg/L group. **, $p < 0.001$; *, $p < 0.01$. *p*-values were from one way ANOVA test.

3.12 Correlation of blood pressure with PUA levels

Since we found the significantly higher levels of PUA and blood pressure (DBP and SBP) in both male and female subjects in arsenic-endemic areas than those in non-endemic area, we next examined the correlations of DBP and SBP with PUA levels. We found that both DBP and SBP showed significant ($p < 0.001$ for DBP in both male and female study subjects, and $p < 0.01$ and $p < 0.05$ for SBP in male and female study subjects, respectively) positive correlations with PUA levels (Table 3.3).

Table 3.4 Associations of blood pressure (DBP and SBP) with PUA and other variables through multiple regression analyses

Independent variable		Dependent variable			
		DBP		SBP	
		Male	Female	Male	Female
PUA	β-Coefficient	1.972	3.697	2.296	4.275
	p-value	< 0.001	< 0.001	< 0.01	< 0.001
BMI	β-Coefficient	0.612	0.749	1.228	1.482
	p-value	< 0.01	< 0.001	< 0.001	< 0.001
Age	β-Coefficient	-0.007	0.174	0.070	0.309
	p-value	0.871	0.011	0.284	0.006
Smoking	β-Coefficient	0.269		0.935	
	p-value	0.823		0.585	

Abbreviations: DBP, Diastolic Blood Pressure and SBP, Systolic Blood Pressure. *p*-values were from multiple regression analysis.

3.14 Comparisons of PUA levels in arsenic-endemic normotensive and hypertensive study subjects

We examined the relationship of the elevated levels of PUA with hypertension in both male and female study subjects in arsenic-endemic areas. We divided arsenic-endemic male and female subjects into two groups: normotensive and hypertensive. Intriguingly the results showed that PUA levels were significantly higher in the hypertensive group in both male and female study subjects compared to the normotensive group.

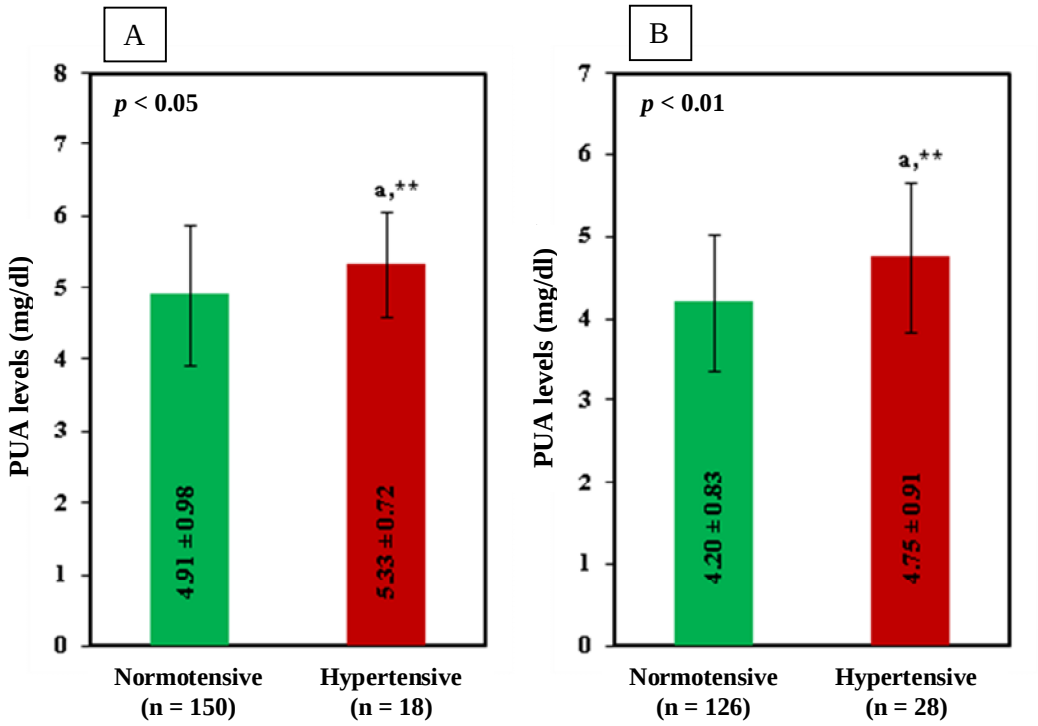


Figure 3.10 PUA levels in arsenic-endemic normotensive and hypertensive group both in male (A) and female (B) study subjects.
[a]Significantly different from normotensive group. **, $p < 0.01$.

3.15 Associations of PUA levels with circulating markers for CVDs

Previously research team of our laboratory reported the associations of arsenic exposure with several cardiovascular markers such as CRP, ICAM-1 and VCAM-1. Association of PUA with hypertension observed in this study led us to analyze the relationship of PUA levels with CRP, ICAM-1 and VCAM-1 among the study subjects who provided blood samples in both the previous and present studies. More than 300 hundred study subjects in this study (n = 305 for CRP; n = 314 for ICAM-1 and VCAM-1) were overlapped with our previous study (Karim et al., 2013). The results showed that PUA levels in the overlapping study subjects showed significant positive correlations with CRP, ICAM-1 and VCAM-1 (Table 3.5). These data suggested that PUA levels were also associated with atherosclerosis-related events among the residents in arsenic-polluted areas.

Table 3.5 Correlations of PUA with CRP, ICAM-1 and VCAM-1

		CRP	**ICAM-1**	**VCAM-1**
PUA	r_s	0.119	0.251	0.139
	p-value	0.038	< 0.001	0.013
	n	305	314	314

n = number of the study subjects overlapped with the previous study (Karim et al. 2013). r_s and *p*- values were from Spearman correlation coefficient test.

Chapter 4

Discussion and Conclusion

Discussion

Arsenic is a potent toxicant and a major public health concern in many countries. Chronic exposure to arsenic is associated with many adverse health effects including CVDs. CVDs are the major causes of chronic arsenic-exposure related morbidity and mortality, however, uncertainties remain in the etiology of arsenic-induced CVDs. Elevated level of blood uric acid is involved in the pathogenesis of gout. Recent advancement in understanding the gout has demonstrated the link between uric acid and CVDs (Abbott et al., 1988; Krishnan et al., 2006, 2008). Epidemiological studies have reported the relationships of blood uric acid levels with several indicators of CVDs (Erdogan et al., 2005; Fukui et al., 2008; Mutluay et al., 2012; Pacifico et al., 2009; Zhang et al., 2012), whereas other studies did not observe such links (Jee et al., 2004; Sakata et al., 2001). It has been argued that the latter studies might not have sufficiently accounted for differences in gender, or for risk factors being strongly related to blood uric acid levels (Storhaug et al., 2013). Recently a series of studies have established uric acid as a surrogate or an independent marker of atherosclerosis, a key event of various forms of CVDs (Bos et al., 2006; Erdogan et al., 2005; Fukui et al., 2008; Ioachimescu et al., 2008; Mutluay et al., 2012; Niskanen et al., 2004; Pacifico et al., 2009; Puig et al., 1999; Storhaug et al., 2013; Zhang et al., 2012). In this study, we found that PUA levels were ($p < 0.001$) significantly higher in arsenic-endemic male and female groups than the non-endemic counterparts (Figure 3.3). Arsenic concentrations in the water, hair, and nails showed significant positive correlations with PUA levels in both male and female study subjects (Figure 3.4 and 3.5). All arsenic exposure metrics showed dose-response relationships with PUA levels (Figure 3.6, 3.7 and 3.8). Further DBP and SBP showed significant positive

associations with PUA levels (Table 3.3), and arsenic-endemic study subjects who were hypertensive had higher levels of PUA compared to the normotensive study subjects (Figure 3.10). The results related to the elevated levels of PUA in arsenic-endemic population with hypertension observed in this study were in good agreement with the previous findings which suggest that elevated level of PUA is associated with hypertension (Jossa et al., 1994; Mutluay et al., 2012).

Uric acid has both prooxidant and antioxidant activities depending on the conditions. As a prooxidant, uric acid causes the oxidation of low density lipoprotein (LDL) (Abuja, 1999; Bagnati et al., 1999). The oxidized LDL (ox-LDL) is one of the key molecules involved in the pathogenesis of atherosclerosis. Furthermore, uric acid can acts as a prooxidant within cells to induce proinflammatory pathways associated with CVDs (Kanellis and Kang, 2005). In our recent study (Karim et al., 2013), we have reported that arsenic exposure is associated with the elevated levels of ox-LDL. However, how arsenic exposure causes the elevation of ox-LDL remains unclear. The elevated levels of PUA in arsenic-endemic individuals observed in this study may at least in part be the possible explanation for the mechanism of arsenic-induced increase in the levels of plasma ox-LDL. Further experimental evidence is required to support this notion.

We have found that arsenic exposure is significantly associated with the increased levels of plasma big endothelin, CRP, ICAM-1 and VCAM-1 which are the markers for endothelial damage or dysfunction (Hossain et al., 2012; Karim et al., 2013). Uric acid, through its prooxidant activity, causes endothelial dysfunction by reacting with and removing nitric oxide (NO), thereby preventing vasodialation of the endothelium.

Decreased NO and increased reactive oxygen species may promote a proinflammatory state that causes endothelial dysfunction, and contributes to atherosclerosis (Johnson et al., 2003). Finally, uric acid inhibits endothelial cell proliferation and stimulates CRP production (Kanellis and Kang, 2005). On the other hand, uric acid can produce CRP through the stimulation of smooth muscle cells. Uric acid stimulates the production of monocyte chemotactant protein-1, a key chemokine implicated in increased cell proliferation and production of CRP. Increased level of CRP is a key indicator of proinflammatory microenvironment toward the atherosclerosis.

In this study, we found that arsenic-endemic hypertensive study subjects had significantly high levels of PUA (Figure 3.10). PUA levels were also found to be significantly associated with blood pressure (DBP and SBP) in multiple regression analyses (Table 3.4). These results supported the involvement of PUA in hypertension in arsenic-endemic individuals. When arsenic exposure metrics were included in multiple regression analyses as independent variables, arsenic concentrations in water, hair and nails showed the highest β-Coefficient, and the values of β-Coefficients of PUA levels were decreased or lost statistical significance in some cases (Table 3.2). This is understandable because the arsenic exposure levels had strong associations with both blood pressure and PUA levels, which resulted in masking of the association of PUA with blood pressure in multiple regression analyses. It seems likely that arsenic exposure caused hypertension or other multiple vascular lesions, which are reflected in the increases in the levels of several biochemical indicators of CVDs including PUA. To further confirm the association of PUA levels with other biochemical indicators related to the development of CVDs, we examined the correlations of PUA levels with CRP, ICAM-1 and VCAM-1. We selected the study

subjects who had also attended the previous study in which plasma levels of CRP, ICAM-1 and VCAM-1 in relation to arsenic exposure were investigated (Karim et al., 2013). Intriguingly, we found that PUA levels for the overlapping study subjects between the present and previous studies had significant positive associations with CRP, ICAM-1 and VCAM-1(Table 3.5). The associations of PUA levels with the biomarkers of CVDs provide evidence supporting the pathophysiologic implication of the increased levels of PUA in hypertension and other forms of CVDs in arsenic-endemic individuals.

In this study, PUA levels were found to be increased dose-dependently in both male and female groups (Figure 3.6, 3.7 and 3.8). PUA levels were significantly higher in the high exposure groups than the low exposure group across the three kinds of exposure metrics (water, hair and nail arsenic concentrations). Furthermore, PUA levels were higher in the >50 μg/L groups as compared to the ≤ 10 μg/L groups in the classification of the study subjects based on the maximum permissive limit of water arsenic set by WHO (10 μg/L) and Bangladesh Government (50 μg/L) in both males and females (Figure 3.9). PUA levels in the 10.1-50 μg/L group in male study subjects were significantly higher than the ≤ 10 μg/L group. These results were particularly important from a policy perspective.

Since the base line levels of PUA are different between males and females, we examined all the associations of arsenic exposure and PUA levels separately in male and females. The association of arsenic exposure with PUA levels was consistent in both males and females, suggesting that arsenic exposure increased the PUA levels irrespective of gender. The average levels of PUA as shown in (Figure 3.3) in arsenic-

endemic and non-endemic populations of both sexes were within the normal range (men: 3.4-7.0 mg/dL and women: 2.4-5.7 mg/dL). Gout is more common in men than women because of the higher base line values of PUA in men than in women. This implies that the subtle increase in the levels of PUA within a normal range among arsenic-endemic individuals may increase the risk of hyperuricemia-related diseases.

Blood PUA has been recognized as a marker of decreased kidney function. To clarify whether the elevated PUA levels in arsenic-endemic individuals observed in this study were caused by renal dysfunction, we measured BUN as a marker of renal dysfunction. As shown in (Table 3.1) we found that the average BUN levels were in normal range in all groups and no significant differences were found between arsenic-endemic and non-endemic male and female subjects. Further, in the multiple regression analyses, BUN levels did not show significant β-Coefficients in male study subjects (Table 3.2). In female study subjects, BUN levels showed significant β-Coefficients, but the β-Coefficients values of BUN were much lower than those of arsenic exposure metrics (Table 3.2). Although BUN is not a single indicator for renal dysfunction, these results suggest that renal dysfunction is not a major cause for the elevated PUA levels in arsenic-endemic individuals. Nevertheless, it should be examined in a future study whether a slight correlation of BUN with PUA observed only in females in multiple regression analyses actually involves biologically significant events.

There were several strengths of this study. 1) This study for the first time demonstrate the effects of arsenic exposure on PUA levels, through monitoring three different exposure metrics (water, hair and nail arsenic levels) in a good number of study

population with a large variation in their arsenic exposure levels. 2) There were strong positive correlations between drinking water and hair arsenic concentrations (r_s = 0.767, $p < 0.001$), and between water and nail arsenic concentrations (r_s = 0.788, $p <$ 0.001), (Figure 3.1 and 3.2). Strong correlations among these three kinds of exposure metrics might remove the possibility of misclassifications. 3) This study showed the positive associations of arsenic exposure with PUA levels in male and female groups separately. To obtain positive and significant associations of arsenic exposure with PUA levels in male and female study subjects was important since base levels PUA in males and females are different. 4) This study also demonstrated a relationship of the elevated levels of PUA with hypertension and circulating markers of CVDs in arsenic-endemic populations (Table 3.3, 3.5 and Figure 3.10). These results indicated the implication of the elevated levels PUA in hypertension and other forms of CVDs. However, there are some limitations of this study that are necessary to be discussed. First, we showed the association between arsenic exposure and PUA levels after adjusting the age, BMI, smoking habits and BUN (Table 3.2). However, other factors such as co-exposure to other metals, insecticides or pesticides or individual variations could influence the association between arsenic exposure and PUA levels. If other factors could influence the observed association, they would also follow the same concentration gradients as arsenic in the drinking water, hair and nails. This is unlikely, but a more extensive study is required in future addressing the involvement of other factors that may influence the relationship between arsenic exposure and PUA levels. Second, most of our study subjects were lean (lower end of the normal range) with regards to BMI (Table 3.1). Third, this study was designed as a cross-sectional, but not a prospective study. Further verification of the cause-effect

relationship between arsenic exposure and PUA levels and its implications in the development of CVDs especially hypertension would require a cohort-based study. Fourth, Choi et al. (2005) reported that higher levels of meat and sea foods consumption rather than the total protein intake are associated with the elevated levels of uric acid and suggested that dairy consumption is inversely associated with uric acid levels. In our questionnaire, we obtained information on the general food items consumed by the study subjects. Food habits of both arsenic-endemic and non-endemic study population were almost similar since their socioeconomic status was similar. Both population groups occasionally eat meat or dairy products. Study populations generally do not eat sea foods since sea foods are not popular to the common people who lives in inland in Bangladesh. Furthermore, sea foods are not available in many areas of the country. Therefore, the effects of meat, dairy products and sea foods on the observed association between arsenic exposure and PUA levels were unlikely, but these factors could not be completely ignored. Fifth, all study subjects were in low socioeconomic conditions (Table 3.1). Thus, the findings of the current study may not be pertinent to other study populations because of the different distribution of risk factors for PUA levels that may influence the effect of arsenic exposure. Nevertheless, the increased PUA levels with the increasing concentrations of arsenic and their correlation with hypertension may be significant for obtaining novel mechanistic insights into the arsenic-induced CVDs or other pathogenesis.

Conclusion

In this study, we for the first time demonstrated the novel associations of arsenic exposure with PUA levels through a cross sectional study. We found that PUA levels were significantly higher in arsenic-endemic individuals than those in non-endemic individuals. Arsenic exposure levels showed significant positive correlations with PUA levels. PUA levels were found to be associated with arsenic exposure metrics dose-dependently. Arsenic-endemic hypertensive study subjects had significantly higher levels of PUA compared to the normotensive groups. PUA levels were found to be positively associated with circulating biomarkers related CVDs. Thus the increased levels of PUA and their associations with hypertension and circulating markers of CVDs observed in this study indicate that elevated levels of PUA may be used as a predictive marker of CVDs in the individuals who are exposed to arsenic chronically.

Chapter 5

Summary of the thesis

5.1 Objectives

Arsenic is a potent environmental pollutant and well established human carcinogen that has caused in environmental tragedy in some parts of the world especially in Bangladesh where millions of people have been affected because of the drinking of water contaminated with higher level of arsenic than the maximum permissive limit (10 µg/L) set by World Health Organization (WHO). It is one of the major threats to the public health in Bangladesh. WHO has described arsenic toxicity in Bangladesh as one of the largest mass poisoning in the history of human civilization. Arsenic exposure is associated with a variety of diseases including cardiovascular diseases (CVDs). CVDs are the major causes of arsenic-related morbidity and mortality. Previous studies have shown that arsenic exposure is associated with hypertension, a common form of CVDs. Recent reports suggest PUA as a putative marker for CVDs. However, the association between arsenic exposure and PUA levels has not yet been elucidated. Therefore, the present study for the first time investigated the relationship of chronic human exposure to arsenic with PUA levels especially in connection with hypertension and circulating markers for CVDs.

5.2 Summary of results

As far as we are aware, this is the first study that demonstrates the associations of arsenic exposure with PUA levels and its relationship with hypertension. A total of 483 human subjects, 322 from arsenic-endemic and 161 from non-endemic areas in Bangladesh were recruited as study subjects. This study demonstrated that arsenic levels in the drinking water of the study subjects were positively associated with hair and nail arsenic concentrations. This results suggest that drinking water was the main contributor to the accumulated arsenic in hair and nail of the study subjects. PUA levels were significantly higher in males and females living in arsenic-endemic areas

than those in non-endemic area. Arsenic exposure (water, hair and nails arsenic concentrations) showed significant positive correlations with PUA levels. In multiple regression analyses, arsenic exposure levels were found to be the most significant contributors on PUA levels among the other variables that included age, body mass index, BUN, and smoking. There were dose-response relationships between arsenic exposure and PUA levels. DBP and SBP also showed significant positive correlations with PUA levels. The average PUA levels were significantly higher in the hypertensive group than those in the normotensive group in both males and females living in arsenic-endemic areas. Intriguingly, we found that PUA levels for the overlapping study subjects between the present and the previous study of our research group (Karim et al., 2013) had significant positive associations with the circulating biomarkers (CRP, ICAM-1, VCAM-1) related to CVDs. Taken together, the results of this study suggest that elevated levels of PUA in arsenic-endemic individuals may be implicated in arsenic-induced CVDs.

5.3 Strengths and limitations

5.3.1 Strengths

There were several unique features in the research.

First, the major strength of this dissertation was to show all the associations of PUA levels across the three kinds of exposure metrics (water, hair and nail arsenic concentrations) in a good number of study populations with a wide variation of arsenic exposure levels. Therefore, the assessment of arsenic exposure by three kinds of exposure metrics and their correlations with PUA levels might exclude the possibilities of miss classification. Second, there were strong positive correlations between drinking water and hair arsenic concentrations, and between water and nail

arsenic concentrations Strong correlations among these three kinds of exposure metrics might remove the possibility of misclassifications, Third, to obtain significant associations of arsenic exposure with PUA levels in male and female study subjects separately was important since base level PUA in males and females are different. Forth, this study demonstrated the possible implications of elevated levels of PUA in arsenic-induced hypertension and CVDs.

5.3.2 Limitations

Despite several major strengths, this study had some limitations warranting further discussion.

Although we showed the association between the arsenic exposure and circulating PUA levels adjusting for relevant covariates, there might be some other factors such as co-exposure to other metals, insecticides or pesticides or even individual food habits that could influence the level of circulating molecules. If any accompanying metals or other contaminants or food items could influence the observed associations, then they would also be expected to follow the same concentration gradients as arsenic in the drinking water, hair and nails. This is unlikely, but more extensive study of the other metals and their associations with PUA levels are required in future. This study was designed to be cross sectional, hence causality was difficult to infer. A cohort based study is needed in future to verify the cause-effect relationship of arsenic exposure with PUA levels. All study subjects were in low socioeconomic conditions. Thus, the results of the current study may not be generalizable to other study populations and the study needs to be replicated in other population.

5.4 Public health relevance

Arsenic is a potent environmental pollutant that has caused an environmental tragedy in some parts of the world especially in Bangladesh where tens to thousands of people have been affected because of the drinking of water contaminated by arsenic. Recent reports suggest that arsenic has entered the food chain including rice and vegetables. Since arsenic enters the food chain, the exposure to arsenic is unavoidable. Arsenic has been associated with several chronic diseases such as dermatitis, variety of cancers, CVDs, diabetes mellitus, liver and kidney dysfunctions. CVDs and cancer are the major causes of arsenic-related mortality in arsenic-endemic areas. CVDs are also the leading causes of death all over the world. Therefore, even small contribution of arsenic in the development of CVDs can cause a huge number of excess deaths. Millions of people have already been affected by arsenic. Additional approximately 80 million are at risk of arsenic poisoning. This is the estimation in the case of Bangladesh. However, there are many other countries where arsenic toxicity has also taken as an endemic form. Therefore, this epidemiological research is completely relevant to the public health of those countries which are now under the threat of arsenic poisoning.

In this study, we measured PUA levels and demonstrated its association with circulating markers related to CVDs. Circulating molecules are important in blood biochemistry to asses or predict the diseases. Increased levels of PUA may be an indicator for the development of CVDs in arsenic-endemic individual. Further, the results of the research may be valuable for the development of awareness among the arsenic-endemic people, policy makers and health workers about the adverse health effects of chronic exposure to arsenic. The results of this study may be important from policy perspective.

This research also stated that arsenic exposure dose-dependently associated with PUA levels. Increased PUA levels were also found to be positively associated with circulating molecules implicated in CVDs. Thus these results of this study shed light on the mechanisms of CVDs. Understanding mechanism are critically important for the prevention and therapeutic intervention of the diseases. Thus, the objectives and findings of this study are very much relevant to the public health concern of Bangladesh and other arsenic-endemic countries of the world.

Chapter 6

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Chapter 7

Publication during the PhD period

Publication during the PhD period

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Elevated levels of plasma uric acid and its relation to hypertension in arsenic-endemic human individuals in Bangladesh

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ABSTRACT

Blood uric acid has been recognized as a putative marker for cardiovascular diseases (CVDs). CVDs are the major causes of arsenic-related morbidity and mortality. However, the association of arsenic exposure with plasma uric acid (PUA) levels in relation to CVDs has not yet been explored. This study for the first time demonstrated the associations of arsenic exposure with PUA levels and its relationship with hypertension. A total of 483 subjects, 322 from arsenic-endemic and 161 from non-endemic areas in Bangladesh were recruited as study subjects. Arsenic concentrations in the drinking water, hair and nails of the study subjects were measured by inductively coupled plasma mass spectroscopy. PUA levels were measured using a colorimetric method. We found that PUA levels were significantly ($p < 0.001$) higher in males and females living in arsenic-endemic areas than those in non-endemic area. Arsenic exposure (water, hair and nail arsenic) levels showed significant positive correlations with PUA levels. In multiple regression analyses, arsenic exposure levels were found to be the most significant contributors on PUA levels among the other variables that included age, body mass index, blood urea nitrogen, and smoking. There were dose–response relationships between arsenic exposure and PUA levels. Furthermore, diastolic and systolic blood pressure showed significant positive correlations with PUA levels. Finally, the average PUA levels were significantly higher in the hypertensive group than those in the normotensive group in both males and females living in arsenic-endemic areas. These results suggest that arsenic exposure-related elevation of PUA levels may be implicated in arsenic-induced CVDs.

Introduction

Arsenic is a potent environmental pollutant and human carcinogen that is ubiquitously present in food, soil, water and airborne particles. People are generally exposed to arsenic through contaminated drinking water, food, and air-dust. Occupational exposure to arsenic may also occur through the inhalation of arsenic dusts in the production and distribution processes. However, contaminated drinking water has been recognized as the major source of human exposure to arsenic (Ali et al., 2010; Smith et al., 2000). Arsenic poisoning is a global problem since arsenic contamination of ground water has been discovered in many countries including Bangladesh, India, Pakistan, Argentina, Mexico, Chile, United States of America, Taiwan and China. Arsenic poisoning has taken a serious turn affecting millions of people in Bangladesh (Smith et al., 2000). Elevated levels of arsenic have been reported in 61 out of 64 districts (administrative blocks) in the country and the scale of disaster has exceeded the Chernobyl catastrophe in Ukraine and Bhopal accident in India (Smith et al., 2000). Many people have died of the chronic diseases caused by prolonged exposure to arsenic. It has been assumed that 80–100 million people are at risk of arsenic poisoning in the country (Caldwell et al., 2003; Chowdhury, 2004; Chowdhury et al., 2000). Ingestion of inorganic arsenic has been documented to be associated with a variety of diseases including cancers, cardiovascular diseases (CVDs), dermatitis, neurotoxicity, diabetes

Abbreviations: BMI, Body Mass Index; BUN, Blood Urea Nitrogen; PUA, Plasma Uric Acid; CRM, Certified Reference Material; CRP, C-reactive Protein; CVDs, Cardiovascular Diseases; DBP, Diastolic Blood Pressure; ICAM-1, Intercellular Adhesion Molecule-1; LDL, Low Density Lipoprotein; Ox-LDL, Oxidized-LDL; SBP, Systolic Blood Pressure; VCAM-1, Vascular Cell Adhesion Molecule-1.

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mellitus, renal failure and liver dysfunction (Guha Mazumder et al., 1998; Islam et al., 2011; Karim et al., 2013; Meliker et al., 2007; Tapio and Grosche, 2006; Vahidnia et al., 2008; Wang et al., 2002).

Uric acid is the end product of purine metabolism in humans, and is excreted mainly in the urine. Hepatic production and renal and gut excretion of this compound occur through complex processes. The endogenous production of uric acid occurs in the liver, intestine, muscles, kidneys and the vascular endothelium. Many enzymes are involved in the conversion of two purine bases in nucleic acid, adenine and guanine to uric acid. The final reactions of uric acid production are the conversion of hypoxanthine to xanthine and then to uric acid by the enzyme xanthine oxidase. Humans cannot oxidize uric acid to the more soluble compound allantoine due to the lack of the enzyme uricase, as is different from the other mammals. Because of the functional mutations during the early stage of hominoid evolution, humans and other primates have no functional uricase, which leads to higher blood uric acid levels when compared to rodents. The plasma uric acid (PUA) levels are varied by multiple factors including environmental and genetic factors (Nath et al., 2007). The elevated level of blood uric acid is associated with gout. Pathologically, the increased levels of PUA lead to the formation of crystal deposits in joints, tendons and other tissues (Becker and Roessler, 1995). Besides the role of uric acid in the development of pathologic gout, however, a growing body of evidence has suggested that hyperuricemia is associated with the risk of CVDs including hypertension, metabolic syndrome, coronary artery disease, vascular dementia, stroke, preeclampsia, and kidney diseases (Cannon et al., 1966; Ford et al., 2007; Lehto et al., 1998; Roberts et al., 2005; Schretlen et al., 2007; Siu et al., 2006; Tuttle et al., 2001). Niskanen et al. (2004) conducted a prospective cohort study and showed that hypeuricemia is an independent risk factor for CVDs in middle-aged men. Furthermore, Storhaug et al. (2013) stated that serum uric acid is an independent marker of ischemic stroke in men, and the all-cause mortality in general Caucasian populations.

Many studies conducted in the arsenic-endemic populations in the world have clearly suggested that arsenic exposure is associated with CVDs (Chen et al., 2011; Karim et al., 2013; Wang et al., 2002). CVDs are the major causes of arsenic-related morbidity and mortality (Chen et al., 2011). Previously we and other groups have showed that arsenic exposure is associated with hypertension, a common form of CVDs (Hossain et al., 2012; Rahman et al., 1999). Although PUA is a putative marker for CVDs, the association between environmental arsenic exposure and PUA levels has not yet been documented. Therefore, the present study has been conducted to assess the relationship of chronic human exposure to arsenic with PUA levels especially in connection with hypertension.

Methods

Study areas and subjects. Ethical permission was taken from the Institute of Biological Sciences, University of Rajshahi, Bangladesh (21/320-IAMEBBC/IBSc). The subjects who participated in this study gave their written consent and all sorts of confidentialities and rights of the study subjects were strictly maintained. Arsenic-endemic and non-endemic study areas for this study were selected as described previously (Ali et al., 2010; Hossain et al., 2012; Islam et al., 2011; Karim et al., 2010). Arsenic-endemic areas were selected from the North-West region of Bangladesh that included Marua in Jessore, Dutpatila, Jajri, Vultie and Kestopur in Chuadanga, and Bheramara in Kushtia district of Bangladesh, and Chowkoli, a village in Naogaon district with no history of arsenic contamination was selected as a non-endemic area. Local residents (15–60 years of ages) who had lived for at least five years in arsenic-endemic and non-endemic areas were recruited for this study.

During the sample collection process, we were blinded to arsenic levels in the drinking water, hair and nails of the study participants. Attempt was made to match, as much as possible the following: age, sex and socioeconomic parameters (occupation, monthly income and education) of arsenic-endemic and non-endemic study subjects. The ratio of endemic to non-endemic subjects was approximately 2:1, and the ratio of male to female was approximately 1:1.

Pregnant and lactating mothers and the individuals who had a history of surgical operation, drug addiction, hepatitis B positive, hepatotoxic and anti-hypertensive drugs, malaria, kalazar, chronic alcoholism, history of hepatic, renal or severe cardiac diseases, and gout have been excluded from this study. Of the 331 individuals who were approached, 9 were excluded according to the exclusion criteria [i.e., study candidates ($n = 4$) who had lived in arsenic-endemic areas for less than 5 years, pregnant and lactating mothers ($n = 3$) and had hepatic diseases ($n = 2$)]; thus a total 322 were finally recruited in arsenic-endemic areas. In non-endemic area 4 [i.e., study candidates ($n = 2$) who had lived in the non-endemic area for less than 5 years, pregnant mother ($n = 1$), study subjects who underwent recent surgical operation ($n = 1$)] from 165 were excluded. The final participants in the non-endemic area were 161.

Household visits were carried out to interview residents. The personal interviews of the study subjects were carried out by the trained members of our research team using a standardized questionnaire. The information obtained from the interview included the sources of water for drinking and daily household uses, water consumption history, socioeconomic status, occupation, food habit, general food items consumed daily, cigarette smoking, alcohol intake, personal and family medical history, history of diseases, physiological complications, major diseases, previous physician's reports, and body mass index (BMI). We collected the blood and other specimens, and water samples on the same day at each site.

Blood pressure measurement. The standard protocol for measuring blood pressure recommended by World Health Organization (WHO) was used in this study. After the study subjects had rested for 20 min or longer, both systolic blood pressure and diastolic blood pressure (SBP and DBP) were measured three times with a mercury sphygmomanometer with subjects sitting. SBP and DBP were defined at the first phase and fifth phase Korotkoff sounds, respectively. The average of three measurements was used for the analysis. Hypertension was defined as a SBP of $\geq$140 mm Hg and a DBP of $\geq$90 mm Hg on three repeated measurements.

Water collection and arsenic analysis. Water samples used as primary sources of drinking water were collected for this study as described by Ali et al. (2010). Water samples from tube wells were collected in acid-washed containers after the well was pumped for 5 min as previously described (Van Geen et al., 2002). Total arsenic concentrations in water samples were determined by inductively coupled plasma mass spectroscopy (ICP-MS), (HP-4500, Agilent Technologies, Kanagawa, Japan) after the addition of a solution of yttrium (10 μg/L in 1.0% nitric acid) as an internal standard for ICP-MS analysis. All samples were determined in triplicate and the average values were used for data analysis. Accuracy of water arsenic measurement was verified using a certified reference material (CRM). "River water" (NMIJ CRM 7202-a No.347 National Institute of Advanced Industrial Science and Technology, Japan) was used as a CRM. The average value (mean $\pm$ SD) of arsenic in the "river water" determined in triplicate by ICP-MS was 1.06 $\pm$ 0.04 μg/L (reference value, 1.18 μg/L).

Collection of hair and nail samples, and analysis of arsenic. Arsenic levels in nails and hair have been reported to provide the integrated measures for arsenic exposure (Agahian et al., 1990; Gault et al., 2008). Hair and nails of the study subjects were collected and washed by the method as described previously (Ali et al., 2010). The washed samples were allowed to dry at 60 °C overnight and digested with concentrated nitric acid using a hot plate at 70 °C for 15 min and 115 °C for 15 min. After cooling, the samples were diluted with 1.0% nitric acid containing yttrium (10 μg/L). The concentrations of arsenic and yttrium

in these samples were determined by ICP-MS. All samples were determined in triplicate and the average values were used. Accuracy of arsenic measurement was verified using "human hair" (GBW09101, Shanghai Institute of Nuclear Research Academia Sinica, China) as a CRM. The average value of arsenic in "human hair" determined in triplicate by ICP-MS was 0.61 ± 0.12 μg/g (reference value, 0.59 μg/g).

Collection of plasma. Fasting blood samples from the study subjects were collected in EDTA-containing blood collection tubes. The blood samples were immediately placed on ice and subsequently centrifuged at 1600 ×*g* for 15 min at 4 °C. The plasma supernatant was taken and stored at −80 °C.

Measurement of PUA and BUN. Both PUA and BUN levels were measured separately by colorimetric methods according to the manufacturer's protocols (Human Diagnostic, Germany) with an analyzer (Humalyzer 3000, USA). All plasma samples were analyzed in duplicate, and the mean values were used.

Statistical analysis. Statistical analysis for this study was performed using the Statistical Packages for Social Sciences (SPSS) software. Characteristics and blood biochemistry data of the male and female study subjects from arsenic-endemic and non-endemic areas were analyzed by independent sample *t*-test and chi-square test. Normality of the distribution of variables was verified by a Q–Q plot. Because of skewed distributions of arsenic exposure metrics, log transformed values were used for statistical analysis. Correlations of PUA levels with arsenic concentrations in the drinking water, hair and nails were evaluated by Spearman correlation coefficient test. Subsequently, multiple regression analyses were performed to examine the associations of PUA levels with arsenic exposure metrics and other variables. The study subjects in the arsenic-endemic areas were stratified through frequency test into 'high' and 'medium' exposure groups based on the concentrations of arsenic in the drinking water, hair, and nails. The study subjects in the non-endemic area were used as a reference group ('low' exposure group). PUA levels in the low, medium and high exposure groups were analyzed by one-way ANOVA followed by Bonferroni multiple comparison tests. Study subjects in arsenic-endemic and non-endemic areas were further divided into three (≤10 μg/L, 10.1–50 μg/L and >50 μg/L) groups based on the regulatory upper limit for water arsenic concentrations set by WHO (10 μg/L) and Bangladesh Government (50 μg/L). PUA levels in the three groups were evaluated by one-way ANOVA (Bonferroni test). Correlations of DBP and SBP with PUA were analyzed by Spearman correlation coefficient test. Multiple regression analyses were performed for the association of blood pressure (DBP and SBP) with arsenic exposure metrics, PUA levels, and other variables. Independent sample *t*-test was performed to compare the PUA levels between normotensive and hypertensive study subjects. Correlations of PUA with C-reactive protein (CRP), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were evaluated by Spearman correlation coefficient test. A value of $p < 0.05$ was considered statistically significant.

Results

General characteristics of the study subjects

General characteristics of the male and female study subjects in the arsenic-endemic and non-endemic areas were shown in Table 1. Of the 483 study subjects, 168 were males and 154 were females from arsenic-endemic areas, and 75 were males and 86 were females from non-endemic area. Arsenic concentrations in the drinking water, hair and nails of the study subjects in arsenic-endemic areas were approximately 70, 19 and 6 times higher in male and 72, 12 and 8 times higher in female groups, respectively than those of non-endemic area. Because attempts were made to match the age, sex, and socioeconomic parameters (occupation, education and monthly income) between arsenic-endemic and non-endemic study subjects, no significant differences were observed in those parameters between the two study groups. The DBP and SBP in arsenic-endemic male and female groups were significantly higher than those of non-endemic subjects. Accordingly, the percentages of hypertensive subjects were also significantly higher in both male and female subjects in arsenic-endemic areas than those in non-endemic area. No significant difference was found in the percentages of tobacco smokers between arsenic-endemic and non-endemic individuals. No females were found to be a smoker. This was expected since Bangladeshi women generally do not smoke cigarettes. None of the study subjects were admitted to drink alcohol. The BMI and BUN levels of both male and female study subjects were almost similar between both arsenic-endemic and non-endemic areas.

Comparison of PUA levels in arsenic-endemic and non-endemic study subjects

Since the base line PUA levels in males and females are different, we compared the PUA levels between arsenic-endemic and non-endemic study populations separately in both sexes. The average PUA levels in both male and female groups in arsenic-endemic areas were significantly ($p < 0.001$) higher than those in non-endemic area (Fig. 1).

Associations of arsenic exposure with PUA levels

Table 2 shows the correlations of PUA levels with arsenic concentrations in the drinking water, hair and nails of the study populations. A significant correlation was observed between PUA levels and arsenic concentrations in the drinking water ($r_s = 0.410$, $p < 0.001$ in male; $r_s = 0.324$, $p < 0.001$ in female). Similar relationships were also observed between PUA and hair arsenic concentrations ($r_s = 0.382$, $p < 0.001$ in male; $r_s = 0.324$, $p < 0.001$ in female), and between PUA and nail arsenic concentrations ($r_s = 0.339$, $p < 0.001$ in male; $r_s = 0.205$, $p < 0.01$ in female). Furthermore, we performed multiple regression analyses to examine the associations of arsenic exposure with PUA levels and other variables (age, BMI, smoking, and BUN). Water, hair, and nail arsenic concentrations showed significant and the highest β-coefficients among the independent variables in both males and females (Table 3). Age, BMI, smoking or BUN did not show significant β-coefficients in males. In females, BUN showed significant β-coefficients, but their values were very low. These data suggested that arsenic exposure had the highest contributions on the elevation of PUA levels even after the adjustment of other confounding factors including BUN.

Dose–response relationships of arsenic exposure with PUA levels

Both male and female study subjects in arsenic-endemic areas were split into two arsenic exposure groups (medium and high) based on each arsenic exposure metrics (water, hair and nail arsenic concentrations), and non-endemic population was used as low or reference group (Table 4). At first we checked the dose–response relationship between external exposure metric (water arsenic concentrations) and PUA levels. Significant differences in PUA levels were found among arsenic exposure groups (ANOVA, $p < 0.001$). Post-hoc Bonferroni multiple comparison test revealed that PUA levels were significantly different for medium versus low, high versus low, and high versus medium exposure groups in both male and female study subjects. Next, we evaluated the dose–response relationship between the internal exposure metrics (hair and nail arsenic concentrations) and PUA levels. Similarly, PUA levels were significantly different among arsenic exposure groups (ANOVA, $p < 0.001$), and post-hoc tests showed that PUA levels were higher in the medium and high exposure groups than the low exposure group in both males and females. Finally we divided the male and

Table 1
General characteristics of the study subjects.

Character	Male			Female		
	Non-endemic	Endemic	*p*-Value	Non-endemic	Endemic	*p*-Value
No.	75	168		86	154	
Age[a]	36.44 ± 12.70	37.52 ± 12.92	0.542*	36.47 ± 10.46	37.29 ± 10.88	0.563*
Water As (μg/L)[b]	0.73 (6.79)	72.75 (6.20)	<0.001*	0.54 (8.12)	68.74 (6.13)	<0.001*
Hair As (μg/g)[b]	0.19 (2.19)	3.00 (2.69)	<0.001*	0.31 (2.19)	2.84 (2.91)	<0.001*
Nail As (μg/g)[b]	0.95 (2.32)	5.81 (2.41)	<0.001*	0.84 (2.32)	6.04 (2.68)	<0.001*
Occupation [n (%)]						
Male						
Farmer	61 (81.33)	139 (82.74)				
Student	6 (8.00)	8 (4.76)	0.483†			
Business	1 (1.33)	4 (2.38)				
Others	7 (9.33)	17 (10.12)				
Female						
Housewives				74 (86.04)	133 (86.36)	
Worker				10 (11.63)	19 (12.34)	0.411†
Student				0	1 (0.65)	
Other				2 (2.33)	1 (0.65)	
Education [n, (%)]						
No formal education	35 (46.67)	83 (49.40)	0.635†	54 (62.79)	85 (55.19)	0.129†
Primary	31 (41.33)	58 (34.52)		29 (33.72)	54 (35.06)	
Secondary	6 (8.00)	21 (12.50)		3 (3.49)	13 (8.44)	
Graduate	3 (4.00)	6 (3.57)		0	2 (1.30)	
Income/month (US$)[a]	26.56 ± 10.19	25.53 ± 11.04	0.491*	22.70 ± 5.43	23.54 ± 9.52	0.382*
DBP (mm Hg)[a]	70.07 ± 8.60	76.46 ± 8.95	<0.001*	69.42 ± 9.81	77.79 ± 12.41	<0.001*
SBP (mm Hg)[a]	110.13 ± 11.24	116.70 ± 13.74	<0.001*	110.35 ± 15.83	121.75 ± 20.58	<0.001*
Hypertension [n, (%)]						
Yes	0	18 (10.71)	<0.01†	3 (3.50)	28 (18.18)	<0.01†
No	75 (100)	150 (89.29)		83 (96.50)	126 (81.82)	
Smoking [n, (%)]						
Yes	27 (36.00)	63 (37.50)	0.823†	0	0	
No	48 (64.00)	105 (62.50)		86 (100)	154 (100)	
Alcohol intake	–	–	–	–	–	–
BMI (kg/m^2)[a]	20.57 ± 2.17	20.03 ± 2.77	0.135*	21.41 ± 3.20	21.34 ± 3.62	0.874*
BUN (mg/dL)[a]	9.67 ± 2.75	9.40 ± 2.30	0.449*	8.34 ± 2.12	8.71 ± 2.51	0.223*

Abbreviations: As, arsenic.
BMI was calculated as body weight (kg) divided by height squared (m^2).
[a] Mean ± SD.
[b] Geometric mean (SD).
* *p*-Values were from independent sample *t*-test.
† *p*-Values were from chi-square test.

female study subjects into three groups (≤10 μg/L, 10.1–50 μg/L and >50 μg/L) based on the regulatory upper limit of water arsenic concentrations set by WHO (10 μg/L) and Bangladesh Government (50 μg/L) in order to evaluate the dose–response relationship of water arsenic concentrations with PUA levels (Table 5). We found that PUA levels were significantly higher in the >50 μg/L groups in both male and female study subjects than the ≤10 μg/L group. Further the 10.1–50 μg/L group showed significantly ($p < 0.001$) higher PUA levels than the ≤10 μg/L group in male but not in female study subjects. PUA levels in the >50 μg/L group of female study subjects were significantly ($p < 0.001$) higher than those of the 10.1–50 μg/L group.

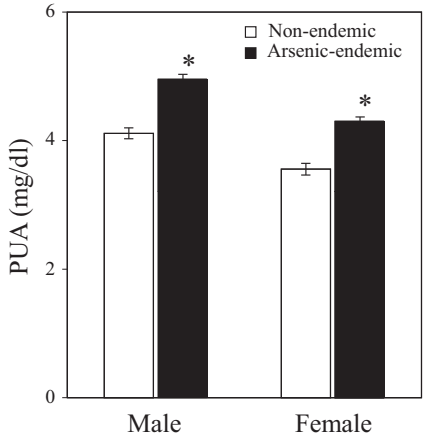

Fig. 1. PUA levels in male and female study subjects in non-endemic and arsenic-endemic areas. White and black columns represent PUA levels (mean ± SE) of the study subjects in non-endemic and arsenic-endemic areas, respectively. *Significantly different at $p < 0.001$. *p*-Values were from the independent sample *t*-test.

Associations of PUA levels with blood pressure

Since we found the significantly higher levels of PUA and blood pressure (DBP and SBP) in both male and female subjects in arsenic-endemic areas than those in non-endemic area, we next examined the correlations of DBP and SBP with PUA levels. As shown in Table 6, both DBP and SBP had significant positive correlations with PUA levels. To explore whether the PUA levels were associated with blood pressure

Table 2
Correlations of arsenic exposure metrics with PUA levels.

Arsenic exposure metrics	Male (n = 243)		Female (n = 240)	
	Correlation coefficient (r_s)	*p*-Value	Correlation coefficient (r_s)	*p*-Value
Water As	0.410	<0.001	0.324	< 0.001
Hair As	0.382	<0.001	0.324	< 0.001
Nail As	0.339	<0.001	0.205	< 0.01

Log-transformed values of arsenic exposure metrics were used. r_s and *p*-values were from Spearman correlation coefficient test.

Table 3
Association of arsenic exposure metrics with PUA levels through multiple regression analyses.

Independent variables	Dependent variable (PUA)				
	Male			Female	
	β-Coefficient	*p*-Value		β-Coefficient	*p*-Value
Water As	0.317	<0.001	Water As	0.176	<0.001
BMI	0.037	0.099	BUN	0.062	0.015
Age	−0.005	0.254	Age	−0.003	0.540
BUN	0.022	0.356	BMI	0.007	0.669
Smoking	0.036	0.764			
Hair As	0.599	<0.001	Hair As	0.410	<0.001
BMI	0.043	0.057	BUN	0.070	0.006
Age	−0.008	0.077	BMI	0.005	0.752
BUN	0.031	0.195	Age	−0.001	0.792
Smoking	0.029	0.811			
Nail As	0.686	<0.001	Nail As	0.265	0.010
BMI	0.044	0.056	BUN	0.072	0.006
Age	−0.006	0.236	Age	−0.003	0.602
Smoking	0.074	0.545	BMI	0.008	0.632
BUN	0.020	0.421			

Log-transformed values of arsenic exposure metrics were used.

after the adjustment with other variables such as age, BMI and smoking, we performed multiple regression analyses using DBP and SBP as dependent variables. As shown in Table 7, PUA levels and BMI showed significant and the highest β-coefficients among the variables both in DBP and SBP. These data suggest that the increased PUA levels had a positive association with blood pressure. When arsenic exposure metrics (water, hair or nail arsenic) were included in this analysis as independent variables, however, all arsenic exposure metrics showed the highest β-coefficients, and those for PUA levels were decreased, and in some cases lost statistical significance (Supplementary Table S1) probably because of highly significant correlations between arsenic exposure metrics and PUA levers as shown in Tables 2 and 3.

Comparison of PUA levels in arsenic-endemic normotensive and hypertensive study subjects

We examined the relationship of the elevated levels of PUA with hypertension in both male and female study subjects in arsenic-endemic areas. We divided arsenic-endemic male and female subjects into two groups: normotensive and hypertensive. The results showed that PUA levels were significantly higher in the hypertensive group in both male and female study subjects compared to the normotensive group (Table 8).

Table 5
Comparisons of PUA levels in three groups based on the regulatory upper limits of water arsenic concentrations set by WHO and Bangladesh Government.

Groups	Male			Female		
	n	PUA (mg/dL)	*p*-Value (F-test)	n	PUA (mg/dL)	*p*-Value (F-test)
≤10 μg/L	98	4.21 ± 0.76	<0.001	114	3.73 ± 0.90	<0.001
10.1–50 μg/L	31	4.81 ± 1.02[a,*]		19	3.73 ± 0.61	
>50 μg/L	114	5.09 ± 0.96[a,**]		107	4.41 ± 0.87[a,**,b,*]	

Data were presented as mean ± SD. Statistically significant association between arsenic exposure levels and PUA levels in one-way ANOVA was examined by F-test, followed by Bonferroni multiple comparisons test between each group of exposure level.
[a] Significantly different from ≤10 μg/L group.
[b] Significantly different from 10.1–50 μg/L group.
** $p < 0.001$.
* $p < 0.01$.

Association of PUA levels with other cardiovascular markers

Previously we reported the associations of arsenic exposure with several cardiovascular markers such as CRP, ICAM-1 and VCAM-1. Association of PUA with hypertension observed in this study led us to analyze the relationship of PUA levels with CRP, ICAM-1, and VCAM-1 among the study subjects who provided blood samples in both the previous and present studies. More than 300 hundred study subjects in this study (n = 305 for CRP; n = 314 for ICAM-1 and VCAM-1) were overlapped with our previous study (Karim et al., 2013). The results showed that PUA levels in the overlapping study subjects showed significant positive correlations with CRP, ICAM-1 and VCAM-1 (Supplementary Table S2). These data suggested that PUA levels were also associated with atherosclerosis-related events among the residents in arsenic-polluted areas.

Discussion

Although it has been well established that chronic exposure to arsenic is associated with CVDs, uncertainties remain in the etiology of arsenic-induced CVDs. Elevated levels of blood uric acid are involved in the pathogenesis of gout. Recent advancement in understanding the gout has demonstrated the link between uric acid and CVDs (Abbott et al., 1988; Krishnan et al., 2006, 2008). Epidemiological studies have reported the relationships of blood uric acid levels with several indicators of CVDs (Erdogan et al., 2005; Fukui et al., 2008; Mutluay et al., 2012; Pacifico et al., 2009; Zhang et al., 2012), whereas other studies did not observe such links (Jee et al., 2004; Sakata et al., 2001). It

Table 4
Dose–response relationship of arsenic exposure metrics with PUA levels.

Exposure metrics	Groups	As levels	Male			Female		
			n	PUA (mg/dL)	*p*-Value (F-test)	n	PUA (mg/dL)	*p*-Value (F-test)
Water As	Low	0.03–13.36	75	4.11 ± 0.74	<0.001	86	3.56 ± 0.84	<0.001
(μg/L)	Medium	0.11–137.35	85	4.78 ± 0.97[a,**]		75	4.11 ± 0.91[a,**]	
	High	140.80–546	83	5.14 ± 0.93[a,**,b,*]		79	4.47 ± 0.79[a,**,b,*]	
Hair As	Low	0.02–1.87	75	4.11 ± 0.74	<0.001	86	3.56 ± 0.84	<0.001
(μg/g)	Medium	0.05–2.86	80	4.89 ± 0.88[a,**]		81	4.19 ± 0.95[a,**]	
	High	2.93–37.24	88	5.01 ± 1.04[a,**]		73	4.42 ± 0.76[a,**]	
Nail As	Low	0.12–8.13	75	4.11 ± 0.74	<0.001	86	3.56 ± 0.84	<0.001
(μg/g)	Medium	0.11–6.34	87	4.90 ± 0.92[a,**]		77	4.29 ± 0.91[a,**]	
	High	6.35–37.42	81	5.02 ± 1.01[a,**]		77	4.31 ± 0.83[a,**]	

Data were presented as mean ± SD. Statistically significant association between arsenic exposure levels and PUA levels in one-way ANOVA was examined by F-test, followed by Bonferroni multiple comparison test between each group of exposure level.
[a] Significantly different from low group.
[b] Significantly different from medium group.
** $p < 0.001$.
* $p < 0.05$.

Table 6
Correlations of blood pressure with PUA levels.

	Male (n = 243)		Female (n = 240)	
	Correlation coefficient (r_s)	p-Value	Correlation coefficient (r_s)	p-Value
DBP	0.223	<0.001	0.257	<0.001
SBP	0.180	<0.01	0.157	<0.05

Abbreviations: DBP, diastolic blood pressure and SBP, systolic blood pressure. r_s and p-values were from Spearman correlation coefficient test.

Table 8
Comparison of PUA levels between arsenic-endemic normotensive and hypertensive study subjects.

Groups	Male			Female		
	n	PUA (mg/dL)	p-Value	n	PUA (mg/dL)	p-Value
Normotensive	150	4.91 ± 0.98	<0.05	126	4.20 ± 0.83	<0.01
Hypertensive	18	5.33 ± 0.72		28	4.75 ± 0.91	

Data were presented as mean ± SD. p-Values were from the independent sample t-test.

has been argued that the latter studies might not have sufficiently accounted for differences in gender, or for risk factors being strongly related to blood uric acid levels (Storhaug et al., 2013). Recently a series of studies (Bos et al., 2006; Erdogan et al., 2005; Fukui et al., 2008; Ioachimescu et al., 2008; Mutluay et al., 2012; Niskanen et al., 2004; Pacifico et al., 2009; Puig and Ruilope, 1999; Storhaug et al., 2013; Zhang et al., 2012) have established uric acid as a surrogate or an independent marker of atherosclerosis, a key event of various forms of CVDs. In this study, we found that PUA levels were significantly higher in arsenic-endemic male and female groups than the non-endemic counterparts (Fig. 1). Arsenic concentrations in the water, hair, and nails showed significant positive correlations with PUA levels in both male and female study subjects (Table 2 and 3). All arsenic exposure metrics showed dose–response relationships with PUA levels (Tables 4 and 5). Further DBP and SBP showed significant positive associations with PUA levels (Tables 6 and 7), and arsenic-endemic study subjects who were hypertensive had higher levels of PUA compared to the normotensive study subjects (Table 8). The results related to the elevated levels of PUA in arsenic-endemic population with hypertension observed in this study were in good agreement with the previous findings which suggest that elevated level of PUA is associated with hypertension (Jossa et al., 1994; Mutluay et al., 2012).

Uric acid has both prooxidant and antioxidant activities depending on the conditions. As a prooxidant, uric acid causes the oxidation of low density lipoprotein (LDL) (Abuja, 1999; Bagnati et al., 1999). The oxidized LDL (ox-LDL) is one of the key molecules involved in the pathogenesis of atherosclerosis. Furthermore, uric acid can act as a prooxidant within cells to induce proinflammatory pathways associated with CVDs (Kanellis and Kang, 2005). In our recent study (Karim et al., 2013), we reported that arsenic exposure is associated with the elevated levels of ox-LDL. However, how arsenic exposure causes the elevation of ox-LDL remains unclear. The elevated levels of PUA in arsenic-endemic individuals observed in this study may at least in part be the possible explanation for the mechanism of arsenic-induced increase in the levels of plasma ox-LDL. Further experimental evidence is required to support this notion.

We have found that arsenic exposure is significantly associated with the increased levels of plasma big endothelin, CRP, ICAM-1 and VCAM-1 (Hossain et al., 2012; Karim et al., 2013) which are the markers for endothelial damage or dysfunction. Uric acid, through its prooxidant activity, causes endothelial dysfunction by reacting with and removing nitric oxide (NO), thereby preventing vasodilation of the endothelium. Decreased NO and increased reactive oxygen species may promote a proinflammatory state that causes endothelial dysfunction, and contributes to atherosclerosis (Johnson et al., 2003). Finally, uric acid inhibits endothelial cell proliferation and stimulates CRP production (Kanellis and Kang, 2005). On the other hand, uric acid can produce CRP through the stimulation of smooth muscle cells. Uric acid stimulates the production of monocyte chemoattractant protein-1, a key chemokine implicated in increased cell proliferation and production of CRP. Increased level of CRP is a key indicator of proinflammatory microenvironment toward the atherosclerosis.

In this study, we found that arsenic-endemic hypertensive study subjects had significantly high levels of PUA (Table 8). PUA levels were also found to be significantly associated with blood pressure (DBP and SBP) in multiple regression analyses (Table 7). These results supported the involvement of PUA in hypertension in arsenic-endemic individuals. When arsenic exposure metrics were included in multiple regression analyses as independent variables, arsenic concentrations in the water, hair, and nails showed the highest β-coefficients, and the values of β-coefficients of PUA levels were decreased or lost statistical significance in some cases (Supplementary Table S1). This is understandable because the arsenic exposure levels had strong associations with both blood pressure and PUA levels, which resulted in the masking of the association of PUA with blood pressure in multiple regression analyses. It seems likely that arsenic exposure caused hypertension or other multiple vascular lesions, which are reflected in the increases in the levels of several biochemical indicators of CVDs including PUA. To further confirm the association of PUA levels with other biochemical indicators related to the development of CVDs, we examined the correlation of PUA levels with CRP, ICAM-1, and VCAM-1. We selected the study subjects who had also attended the previous study in which plasma levels of CRP, ICAM-1, and VCAM-1 in relation to arsenic exposure were investigated (Karim et al., 2013). Intriguingly, we found that PUA levels for the overlapping study subjects between the present and previous studies had significant positive associations with CRP, ICAM-1 and VCAM-1(Supplementary Table S2). The associations of PUA levels with the biomarkers of CVDs provide evidence supporting the pathophysiologic implication of the increased levels of PUA in hypertension and other forms of CVDs in arsenic-endemic individuals.

In this study, PUA levels were found to be increased dose-dependently in both male and female groups (Table 4). PUA levels were significantly higher in the high exposure groups than the low exposure group across the three kinds of exposure metrics (water, hair and nail arsenic concentrations). Furthermore, PUA levels were higher in the >50 μg/L groups as compared to the ≤10 μg/L groups in the classification of the study subjects based on the maximum permissive limit of water arsenic set by WHO (10 μg/L) and Bangladesh Government (50 μg/L) in both males and females (Table 5). PUA levels in the 10.1–50 μg/L group in male study subjects were significantly higher than the ≤10 μg/L group. These results are particularly important from a policy perspective.

Since the base line levels of PUA are different between males and females, we examined all the associations of arsenic exposure and PUA levels separately in male and females. The association of arsenic exposure with PUA levels was consistent in both males and females, suggesting that arsenic exposure increased the PUA levels irrespective of

Table 7
Associations of blood pressure (DBP and SBP) with PUA and other variables through multiple regression analyses.

Independent variable		Dependent variable			
		DBP		SBP	
		Male	Female	Male	Female
PUA	β-coefficient	1.972	3.697	2.296	4.275
	p-Value	0.001	<0.001	0.007	0.001
BMI	β-coefficient	0.612	0.749	1.228	1.482
	p-Value	0.007	<0.001	<0.001	<0.001
Age	β-coefficient	−0.007	0.174	0.070	0.309
	p-Value	0.871	0.011	0.284	0.006
Smoking	β-coefficient	0.269		0.935	
	p-Value	0.823		0.585	

gender. The average levels of PUA as shown in Fig. 1 in arsenic-endemic and non-endemic populations of both sexes were within the normal range (men: 3.4–7.0 mg/dL and women: 2.4–5.7 mg/dL). Gout is more common in men than women because of the higher base line values of PUA in men than in women. This implies that the subtle increase in the level of PUA within a normal range among arsenic-endemic individuals may increase the risk of hyperuricemia-related diseases.

Blood PUA has been recognized as a marker of decreased kidney function. To clarify whether the elevated PUA levels in arsenic-endemic individuals observed in this study were caused by renal dysfunction, we measured BUN as a marker of renal dysfunction. As shown in Table 1, we found that the average BUN levels were in normal range in all groups, and no significant differences were found between arsenic-endemic and non-endemic male and female subjects. Further, in the multiple regression analyses, BUN levels did not show significant β-coefficients in male study subjects (Table 3). In female study subjects, BUN levels showed significant β-coefficients, but the β-coefficient values of BUN were much lower than those of arsenic exposure metrics (Table 3). Although BUN is not a single indicator for renal dysfunction, these results suggest that renal dysfunction is not a major cause for the elevated PUA levels in arsenic-endemic individuals. Nevertheless, it should be examined in a future study whether a slight correlation of BUN with PUA observed only in females in multiple regression analyses actually involves biologically significant events.

The major strengths of this study were 1) to show for the first time the effects of arsenic exposure on PUA levels, through monitoring three different exposure metrics (water, hair and nail arsenic levels) in a good number of study population with a large variation in their arsenic exposure levels, 2) to show the associations of arsenic exposure with PUA levels in male and female groups separately, and 3) to demonstrate a relationship of the elevated levels of PUA with hypertension in arsenic-endemic populations. However, there are some limitations of this study that are necessary to be discussed. First, we showed the association between arsenic exposure and PUA levels after adjusting the age, BMI, smoking habits, and BUN (Table 3). However, other factors such as co-exposure to other metals, insecticides or pesticides or individual variations could influence the association between arsenic exposure and PUA levels. If other factors could influence the observed association, they would also follow the same concentration gradients as arsenic in the drinking water, hair and nails. This is unlikely, but a more extensive study is required in the future addressing the involvement of other factors that may influence the relationship between arsenic exposure and PUA levels. Second, most of our study subjects were lean (lower end of the normal range) with regard to BMI. Third, this study was designed as a cross-sectional, but not a prospective study. Further verification of the cause–effect relationship between arsenic exposure and PUA and its implications in the development of CVDs especially hypertension would require a cohort-based study. Fourth, Choi et al. (2005) reported that higher levels of meat and sea food consumption rather than the total protein intake are associated with the elevated levels of uric acid, and suggested that dairy consumption is inversely associated with uric acid levels. In our questionnaire, we obtained information on the general food items consumed by the study subjects. Food habits of both arsenic-endemic and non-endemic study population were almost similar since their socioeconomic status was similar. Both population groups occasionally eat meat or dairy products. Study populations generally do not eat sea foods since sea foods are not popular to the common people who lives inland in Bangladesh. Furthermore, sea foods are not available in many areas of the country. Therefore, the effects of meat, dairy products and sea foods on the observed association between arsenic exposure and PUA levels were unlikely, but these factors could not be completely ignored. Fifth, all study subjects were in low socioeconomic conditions. Thus, the findings of the current study may not be pertinent to other study populations because of the different distributions of risk factors for PUA levels that may influence the effect of arsenic exposure. Nevertheless, the increased PUA levels with the increasing concentrations of arsenic and their correlation with hypertension may be significant for obtaining novel mechanistic insights into the arsenic-induced CVDs or other pathogenesis.

Conclusions

In this study, we for the first time demonstrated the novel associations of arsenic exposure with PUA levels through a cross sectional study. We found that PUA levels were significantly higher in arsenic-endemic individuals than those of non-endemic individuals. Arsenic exposure levels showed significant positive correlations with PUA levels. PUA levels were found to be associated with arsenic exposure metrics dose-dependently. Further arsenic-endemic study subjects who were hypertensive had significantly higher levels of PUA compared to the normotensive groups suggesting that arsenic exposure-related elevation of PUA levels might be implicated in CVDs. Thus the increased levels of PUA may be used as a predictive marker for CVDs in arsenic-endemic individuals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2014.09.011.

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Annexure

Annexure-I

Questionnaires to the study subject (personal information)

(Confidential)

Research Conducted by: ……

Department of Biochemistry and Molecular Biology, University of Rajshahi.

Subject/patient ID: ……

Place of sample collection:……..

Date of data collection: ……

PERSONAL INFORMATIONS

1. **Name of the subject**: ……
2. Father's /Husband's / Spouse's Name:
   …….....
3. Address: ……..
4. Tel no or mobile no: (if any):
   ……
5. Age: ……
6. Sex: i) M ii) F
7. Occupation: ……
8. Body weight: …….. (in Kg)
9. Body height: ……. (in m) …….. (in ft)
10. Blood pressure (mm Hg): ……
11. Marital status: i) Yes ii) No
12. Members are in the family: i) One ii) Two iii) Three ii) Four iv) Five v) More

13. Socioeconomic conditions:
    Monthly income: i) Individual income ii) House hold income iii) others
    Monthly average income (In taka):

14. Education level: i) No formal education ii) Primary iii) Secondary iv) Higher secondary v) Graduate level

15. Housing Status:
    a) Kacha (Mud with straw roof)
    b) Mud with corrugated tin (roof)
    c) Tin (wall) with corrugated tin (roof)
    d) Brick with corrugated tin (semi Pakka)
    e) Brick with concrete roof (Pakka)

16. Sanitation: i) Yes ii) No
    If yes, i) Kacha (slab with straw or chat or bamboo wall) ii) Semi pakka
    iii) Pakka

17. Television: i) Yes ii) No

INFORMATION RELATED TO ARSENIC EXPOSURE

1. How many members in the family have been affected by arsenic?
   ……

2. Relationship of the arsenic-affected family members with the subject:
   i) Father ii) Mother iii) Husband iv) Brother v) Sister vi) Son vii) Daughter viii) Wife ix) Others
   (specify)……...

3. What are the age and sex of children:
   i) 1st child……. sex: a) M b) F
   ii) 2nd child ……sex: a) M b) F
   iii) 3rd child…….sex: a) M b) F

4. From when symptoms of arsenicosis have been developed in the child?
   ……..……

5. How long he/she is residing in the study area?
   i) 1 year ii) 2 year iii) 5 years iv) More (specify the year)…....

6. Drinking water sources of the study subject: i) Tube-Well ii) Kua
   Is the drinking water contaminated? i) Yes ii) No iii) Not confirmed.

7. If yes, from when the individual came to know? .. ……Years

8.a) Has the drinking water source been checked for arsenic contamination?

i) Yes ii) No iii) unknown

b) Has the tube well marked red? i) Yes ii) No

9. How long did he/she drink water from that source? ……..…….…… years.

10. Major Symptoms and duration of symptoms:

(Specify the symptoms) …….…….

a) Skin (specify the symptoms):

i) Melanosis ii) Hyperkeratosis iii) Both

b) Respiratory complications (specify the symptoms):

i) Asthma ii) COPD iii) DPLD iv) Cough v) Haemoptysis v) SOB vi) Chest pain

c) Urinary related problems (specify the symptoms):

…….……

d) Eye related problem (specify the symptoms):

…….……

e) Diabetes: …….……..

f) Neural problem (specify the symptoms):

…….……..

g) Taste (decrease, increase or unknown): ……

h) Cardiovascular system (specify the symptoms):

i) IHD ii) Hypertension iii) Heart failure

j) Hair loss: …….……

k) Allergy (specify the symptoms): …….……

l) Hearing Problem: ...…….……..

m) Others problems: ……...

11. a) Has the subject already gone to the physician? i) Yes ii) No

If yes, for what problem?

b) Did the physician give you any medicine? i) Yes ii) No iii) Unknown

c) What types of medicine (specify the drugs)? .....

d) Did the physician give you any medicine for the treatment of arsenicosis

i)Yes ii) No iii) Unknown

e) What types of medicine (specify the drugs)? ……...

12. Has any agencies/person checked arsenic level in the food/vegetables/fishes which are consumed by subject? i) Yes ii) No

(If yes, please specify the types of food which contain high level of arsenic)

……

FOOD HABITS AND FOOD FREQUENCY QUESTIONNAIRES:

1. Food taken by the study subjects per day? i) Two ii) Three iii) More

2. Breakfast

a) What are the menus?

……

b) Does the subject eat egg in breakfast? i) Daily ii) Weekly iii) Occasionally

c) What are major items in breakfast? i) Rice ii) Ruti iii) others

d) Name of the rice:

e) Amount of rice:

f) Amount of wheat flour:

3. Lunch

a) Menus: ……...

i) Rice ii) Ruti iii) Others

b)Name of the rice:

c) Amount of rice:

d) Amount of wheat flour:

e) Fish: i) Daily ii) Weekly iii) Occasionally

f) Meat: i) Daily ii) Weekly iii) Occasionally

g) Egg : i) Daily ii) Weekly iii) Occasionally

h) Dal: i) Daily ii) Weekly iii) Occasionally

i) Name of the most frequently consumed dal: i) lentil ii) Mash Kolai iii)Mug iv) Others

j) Vegetables: i) Daily ii) Weekly iii) Occasionally

Name of the Vegetables: ……..…….

4. Dinner

a) Fish: i) Daily ii) Weekly iii) Occasionally

b) Meat: i) Daily ii) Weekly iii) Occasionally

c) Egg : i) Daily ii) Weekly iii) Occasionally

d) Dal: i) Daily ii) Weekly iii) Occasionally

e) Name of the most frequently consumed dal: i) lentil ii) Mash Kolai iii) Mug iv) Others

f) Vegetables: i) Daily ii) Weekly iii) Occasionally

Name of the Vegetables: ……

5. Snacks (if any)

Menus: ...……

a) Tea: i) cup ii) 2 cup iii) 3 cup iv) more

b) Fruits: i) Daily ii) Weekly iii) Occasionally

Types of Fruits: ……...

6. Method of cooking (rice)

i) Complete evaporation (dry) ii) Not evaporation (Marh discarded after cooking)

a) Source of water for cooking: i) Tube-well ii) Well (Kua) iii) others

b) Is the sources of drinking and cooking water same? i) Yes ii) No

c) If No, specify the both sources:

i) Drinking…… ii) Cooking……

d) How much water is used for per kg rice cooking: ...……

e) Name of the family members who eats cooked rice:

i) ……...……

ii) ……...……

iii) ……...……

iv) ...……

v) ...……

7. a) Smoking: i)Yes ii) No

   If yes, how long: …….. and how many cigarette per day ...…….

   b) Betel leaf (pan): i)Yes ii) No

   If yes, how long: ……. and how many time per day: ..……

   c) Betel leaf with chewing tobacco: i) Yes ii) No ii) Alapatha: i)Yes ii) No

8. Is there any visible malnutrition observed in the patients? i) Yes ii) No

   If yes, please specify your observation: ……..……

9. Do you eat sea fish or vegetables? i) Yes ii) No

   If yes, mention the name of vegetables or fish:

   ……

Additional comments: (If any)

……..…

……..…

……..…

Thanks for your participation and cooperation.

Name & Signature of the Investigator (s):

Date: