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Investigation of Association of the Arsenic Exposure with Circulating Molecules Related to Bronchial Asthma

Hossain, Md. Imam

University of Rajshahi

INVESTIGATION OF ASSOCIATION OF THE ARSENIC EXPOSURE WITH CIRCULATING MOLECULES RELATED TO BRONCHIAL ASTHMA

PhD Thesis

A dissertation submitted to the University of Rajshahi in conformity with the requirements for the degree of Doctor of Philosophy in Biochemistry and Molecular Biology

Md. Imam Hossain
Roll No. 11332
Registration No. 1544
Session: 2011-2012

Department of Biochemistry and Molecular Biology
University of Rajshahi, Rajshai -6205, Bangladesh

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Certificate

We certify that the thesis entitled **"Investigation of association of the arsenic exposure with circulating molecules related to bronchial asthma"** submitted by Mr. **Md. Imam Hossain**, incorporates the original research work carried out by him in the Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh under our supervision. We are 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.

(Professor Dr. Md. Khaled Hossain)
Principal Supervisor
Department of Biochemistry and Molecular Biology
University of Rajshahi, Bangladesh

(Professor Dr. Farjana Nikkon)
Co-Supervisor
Department of Biochemistry and Molecular Biology
University of Rajshahi, Bangladesh

Declaration

I hereby declare that the material embodied in this entitle **"Investigation of association of the arsenic exposure with circulating molecules related to bronchial asthma"** prepared for submission in the Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh, for the degree of Doctor of Philosophy. The work contained in this thesis is original and have not been previously submitted anywhere for the awards of any degree.

(Md. Imam Hossain)

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Author

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ABSTRACT

Chronic exposure to arsenic is a major threat to the public health worldwide, affecting hundreds of millions of people. Bangladesh has been grappling with the largest mass poisoning of a population in history because of the contamination of drinking water by inorganic arsenic greater than the permissive limit set by World Health Organization (WHO). Arsenic exposure has been reported to be associated with several chronic diseases including asthma. Asthma is a substantial public health problem among children and adults worldwide. There has been a sharp increase in the global prevalence, morbidity, mortality, and economic burden associated with asthma. Asthma is largely mediated by allergic reactions involving immunoglobulin E (IgE) and Interleukin 4 (IL-4). Although arsenic exposure has been reported to be associated with asthma, however, underlying mechanism linking arsenic exposure and molecules related to asthma has not yet been documented clearly. Therefore, this study was designed to explore the associations of arsenic exposure with serum IgE and IL-4 levels recruiting human populations from arsenic-endemic and non-endemic areas in Bangladesh. Non-endemic subjects were selected from a village in the northern area of Bangladesh with no history of arsenic exposure, and arsenic-endemic subjects were selected from the arsenic-contaminated north-west region of Bangladesh. Drinking water, hair, nail and blood specimens of the study populations were collected for the subsequent laboratory analysis. Arsenic levels in the drinking water, hair and nails of the study subjects were determined by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS). Serum IgE and IL-4 levels were measured by commercially available ELISA kits. In this study, we found that the levels (Mean ± SE) of serum IgE and IL-4 in arsenic-endemic population were 1372.55 ± 96.74 IU/ml and 39.45 ± 1.30 pg/ml, respectively whereas these were 723.08 ± 73.57 IU/ml and 33.63 ± 1.81 pg/ml, respectively in non-endemic population. The differences of serum IgE and IL-4 levels between arsenic-endemic and non-endemic populations

were statistically significant ($p < 0.001$ for IgE and $p < 0.05$ for IL4). Further serum IgE and IL-4 levels were found to be significantly ($p < 0.001$, $p < 0.001$ and $p < 0.01$, respectively for IgE with drinking water, hair and nail arsenic, and $p < 0.01$, $p < 0.01$ and $p < 0.01$, respectively for IL-4 with drinking water, hair and nail arsenic) associated with arsenic exposure metrics. Multiple regression analyses showed that only arsenic concentrations in drinking water, hair and nails but not other variables (age, sex, BMI, smoking and socioeconomic conditions) were significantly associated with serum IgE and IL-4 levels. Further, drinking water, hair and nail arsenic showed dose-response relationships with serum IgE and IL-4 levels. Intriguingly, serum IL-4 levels showed a significant ($p < 0.01$) positive association with circulating IgE and vascular cell adhesion molecule-1 (VCAM-1). Taken together, the results indicate that arsenic exposure-related asthma may be mediated through IgE-IL-4 sensitive pathways.

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ACRONYMS, ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of Variance
As	Arsenic
ATSDR	Agency for Toxic Substances and Disease Registry
BGS	British Geological Survey
BMI	Body Mass Index
CAT	Chronic Arsenic Toxicity
CI	Confidence Interval
CRM	Certified Reference Material
CVD	Cardiovascular Disease
DMA	Dimethylarsinic Acid
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HRP	Horseradish Peroxidase
HSDB	Hazardous Substance Data Bank
IARC	International Agency for Research on Cancer
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IL	Interleukin
IL-4	Interleukin-4
IU	International Unit
MCL	Maximum Contamination Level
MCP	Membrane Cofactor Protein
mg	Milligram

ml	Milliliter
µg	Microgram
MMA	Monomethylarsonic Acid
MRL	Minimum Risk Levels
NIH	National Institute of Health
NRC	National Research Council
PDGF	Platelet-derived Growth Factor
pg	Pico gram
ppb	Parts Per Billions
ppm	Parts Per Millions
ROS	Reactive Oxygen Species
r_s	Spearman's rank correlation coefficient
SD	Standard Deviation
SE	Standard Error
SPSS	Statistical Package for the Social Sciences
$\mathbf{T_H1}$	T-cell subset 2
$\mathbf{T_H2}$	T-cell subset 1
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor Necrosis Factors
UNICEF	United Nations International Children's Emergency Fund
US EPA	United Nations Environmental Protection Agency
USA	United States of America
VCAM-1	Vascular Cell Adhesion Molecule-1
WHO	World Health Organization

Dedicated to my parents....

Chapter I

INTRODUCTION/LITERATURE REVIEW

CHAPTER 1: INTRODUCTION/LITERATURE REVIEW

1.1 Background

The metalloid arsenic is an environmental contaminant and considered as one of the most notorious poisons. It is not clear when and who discovered it. Arsenic has a long history of use as a homicidal agent by ancient Greeks, Persians, Romans and Chinese but in the past 100 years arsenic has been used as a pesticide, a chemotherapeutic agent and a constituent of consumer products (Hughes et al., 2011). Arsenic is ubiquitously present in rocks, soils, water and air. In fact, it is one of the most common elements on earth (Saldivar and Soto, 2009) that ranks 20^{th} in abundance in the earth's crust, 14^{th} in the water, and 12^{th} in the human body (Mandal and Suzuki, 2002). A huge number of populations from the different parts of the world are at risk for poisoning because of the exposure to arsenic through drinking water, air, food, occupation and other environmental sources (Mandal and Suzuki, 2002; Roy and Saha, 2002; Tchounwou et al., 1999). Arsenic can exist in both organic and inorganic forms in the environment. Generally arsenic is found as organic form in different types of foods that are less toxic than the inorganic arsenic (Duxbury et al., 2003; Meharg and Rahman, 2003; Sun et al., 2008), however recent report suggests that many food items from the arsenic-endemic areas also contain inorganic form of arsenic (Huq et al., 2006; Roychowdhury et al., 2004 ; Silbergeld, 2004). Most poisonous form of inorganic arsenic is found in the drinking water (IARC, 2004; Zheng et al., 2004). There are around 200 million people (NRC, 2001) in the world currently at risk for the adverse health effects associated with high concentrations of arsenic in their drinking water, a number which is expected to be further increased due to the recent lowering of limits of arsenic concentration in drinking water to 10 µg/L (Ravenscroft et al., 2009). As a consequence of the biggest arsenic catastrophe has emerged in several parts of the world, the overall situation of arsenic toxicity is alarming. The uses of arsenic-contaminated ground water for irrigation, bioavailability of arsenic to food crops and subsequent consumption by human population and livestock through the food chain have opened additional pathways for arsenic exposure all over the world (Huq et al., 2006). Long-term or chronic exposure to arsenic is associated with dermatitis, cardiovascular diseases, diabetes mellitus,

chronic bronchitis, immune disorders, peripheral neuropathy, liver damage, renal failure, adverse reproductive outcomes, hematological effects, respiratory complications and other ailments (Ali et al., 2010; Argos et al., 2010; Karim et al., 2013; Hossain et al., 2012; Huda et al., 2014; Chen et al., 2007; Mazumder et al., 1998, 2000; Mazumder, 2005; Meliker et al., 2007; Mumford et al., 2007; Tapio and Grosche, 2006; Vahidnia et al., 2008; Wang et al., 2002). In fact, arsenic affects almost all vital organs of human body resulting the damage or dysfunction. Along with the adverse health effects, arsenic toxicity has also created social and economical problems for the residents in the arsenic-endemic areas.

1.2 Physico-chemical properties of arsenic

Arsenic is a chemical element with symbol As and atomic number 33. It is found to exist in many minerals, usually in conjunction with oxygen, chlorine, sulphur and metals, and also as a pure elemental crystal with an atomic weight of 74.92. Arsenic is a metalloid as it has properties of both metals and non-metals. It can exist as powder, amorphous or vitreous forms. Elemental arsenic has a specific gravity of 5.73 sublimes at 615°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. Arsenic trioxide, the most common arsenic compound in commerce, melts at 312°C and boils at 465°C. Elemental arsenic does not dissolve in water; however some salts of arsenic dissolve in water. Further arsenic trioxide, arsenic pentoxide and other arsenical compounds are soluble, depending on the pH and the ionic environment of the solution. When heated to decompose, arsenic compounds emit toxic arsenic fumes (HSDB, 2003). Arsenic can exist in four valence states: –3, 0, +3 and +5. Under reducing conditions, the +3 valence state as arsenite is the dominant form; the +5 valence state as arsenate is generally the more stable form in oxygenized environments (NRC, 1999). Inorganic arsenite and arsenate are the major arsenic species in natural water. Arsenic does react with hot acids to form arsenous acid (H_3AsO_3) or arsenic acid ($H_3\,AsO_4$).

Table 1.1 Chemical nature of arsenic at a glance

Atomic number	33
Atomic mass	74.92 g/mol
Electronegativity (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)

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

1.3 Sources of arsenic

Arsenic is ubiquitously present in food, soil, water and air, and it is released into the environment from both natural and man-made sources. Globally, natural emissions of arsenical compounds have been estimated at about 8,000 tons each year whereas anthropogenic emissions are about 3 times higher (NRC, 1999, 2001). Arsenic is found to exist in several forms in different foods and environmental media such as soil, air, and water. Inorganic arsenic is the predominant form in drinking water, which is both highly toxic and readily bio-available (NRC, 1999, 2001). Arsenicals have been used in various purposes such as medicines, electronics, agriculture, military and metallurgy (Nriagu and Azcue, 1990). Arsenic is also found to exist in the combination with other elements such as sulphur, oxygen and different metals.

1.3.1 Natural sources of arsenic

Arsenic is widely dispersed element in the Earth's crust and found to exist at an average concentration of approximately 5 mg/kg (Garelick et al., 2008). It occurs in trace quantities in all rock, soil, water and air. More than 200 mineral species contain arsenic, the most common of which is arsenopyrite. About one-third of the atmospheric flux of arsenic is of natural origin. Volcanic action is the most important natural source of arsenic followed by arsenic-containing vapour that is generated from solid or liquid forms of arsenic salts at low temperatures. Arsenic is usually concentrated in sulphide-bearing mineral deposits especially those associated with gold mineralization and has a strong affinity for pyrite, one of the more ubiquitous minerals in the earth's crust. It is also concentrated in hydrous iron oxides. Weathering of rocks converts arsenic sulphides to arsenic trioxide, which enters the arsenic cycle as dust or by dissolution in rain, river or groundwater. Arsenic can also enter into the food chain, causing widespread distribution throughout the plant and animal kingdoms. Elevated concentrations of inorganic arsenic (>1 mg/L) in ground water of geochemical origins have also been found in Taiwan (Chen et al., 1985), India (Das et al., 1996; Mandal et al., 1996) and in major parts of Bangladesh (Biswas et al., 1998; Dhar et al., 1997). Organic arsenic compounds such as arsenobetaine, arsenocholine, arsenosugars, tetramethylarsonium salts, and arsenic-containing lipids are mainly found in marine organisms although some of these compounds have also been found in terrestrial species (Francesconi and Edmonds 1997; Grotti et al., 2008).

1.3.2 Man-made sources of arsenic

Commercially elemental arsenic is produced from arsenic trioxide (As_2O_3) by the reduction with charcoal. Arsenic trioxide is a by-product of metal smelting operations. About 70% of the world production of arsenic is used in the timber treatment as copper chrome arsenate (CCA), 22% in agricultural chemicals and the remainder in glass, pharmaceuticals and metallic alloys [(WHO, 2001, http://whqlibdoc.who.int/ehc/WHO_EHC_224.pdf]. Mining, non-ferrous metals smelting and burning of fossil fuels are the major industrial processes that contribute

to arsenic contamination of air, water and soil. Historically, use of arsenic-containing pesticides has left large areas of agricultural land contaminated. Since arsenic is used in the preservation of timber, it also leads to the contamination of the environment. In addition, the use of arsenic-contaminated ground water for irrigation leads to widespread contamination of land and additional exposure to human and livestock via food (Kile et al., 2007; Lindberg et al., 2006; Meharg and Rahman, 2003) all over the world.

1.4 Transformation and mobilization of arsenic in the environment: the arsenic cycle

The principal natural reservoirs of arsenic are rocks where arsenic remains as arsenopyrite (FeAsS). Release and mobilization of arsenic from these sources constitute the availability of this element in soil, water and air in various forms. Under normal ecological conditions, soils may contain arsenic levels between 0.1 and 40 ppm, if the underlying bedrock is not disturbed or redistributed by natural or pedogenic processes (Yan-Chu, 1994). Chemical reactions (i.e. oxidation reduction and methylation) in the soil–water and sediment–rock systems influence the environmental transport, distribution and availability of arsenic. Slow release of arsenic from rocks and sediments or oxidative dissolution of arsenopyrite (FeAsS) from sediments contributes flux of arsenic in the environment. Oxygen availability controls the arsenate–arsenite redox reactions. Adsorption and precipitation of arsenate and arsenite immobilize the soluble arsenic. Methylation of arsenite to monomethylarsonic acid (MMA) or dimethylarsinic acid (DMA) followed by other organoarsenic compounds, constitute the major biological reactions in the arsenic cycle (Figure 1.1) (Bhumbla and Keefer, 1994; Carter and Fairlamb, 1993; Ferguson and Gavis, 1972, Knowles and Benson 1983; Yan-Chu, 1994)

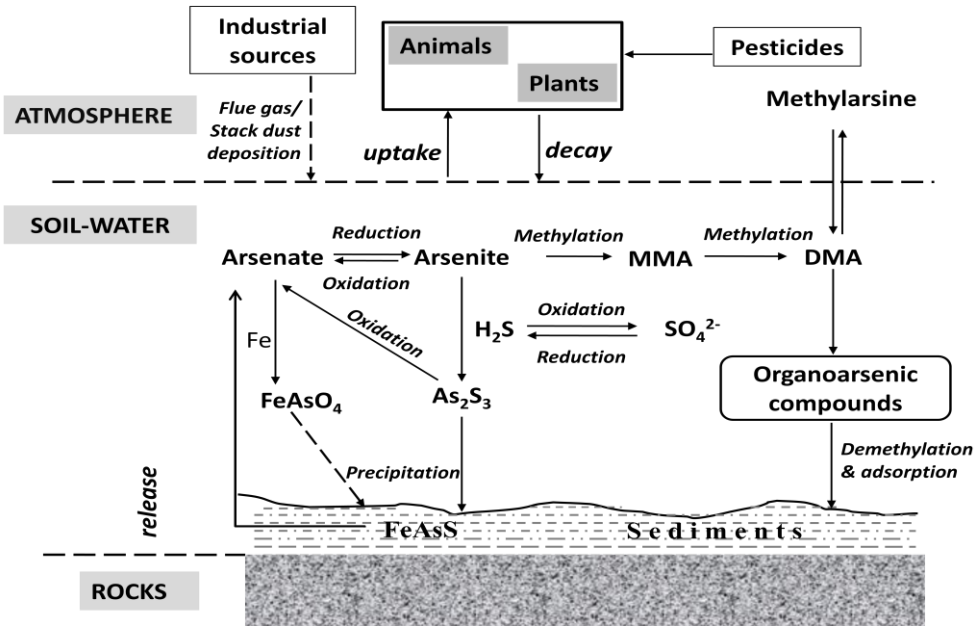

Figure 1.1 Transformation and mobilization of arsenic in the environment

Source: [Roy and Saha, 2002]

1.5 Exposure to arsenic

Human exposure to elevated levels of inorganic arsenic occurs mainly through the consumption of groundwater containing high levels of inorganic arsenic, food prepared with this water and food crops irrigated with high-arsenic water sources (WHO, 2010). Occupational exposure to arsenic occurs in the copper or lead smelting, wood treating, or pesticide application. Workers involved in the production or application of arsenic containing pesticides may also be exposed to higher levels of arsenic. Since arsenic is a natural part of our environment, environmental exposure to arsenic is unavoidable. Various routes of human exposure to arsenic have shown in Figure 1.2. A person can come in contact with arsenic in any of the following ways-

1.5.1 General exposure

Consumption of arsenic through drinking water is the main cause of human exposure to arsenic worldwide. However, high levels of arsenic have been found in the food items collected from the highly arsenic-contaminated areas in the world,

which have been recognized as another potential source of human exposure to arsenic (Huq et al., 2006).

1.5.2 Exposure from drinking water

Drinking water is an important source of exposure to the inorganic arsenic for the people living in areas where arsenic is naturally high in drinking water. In fact, drinking water accounts for most human arsenic exposures worldwide. Arsenic may enter lakes, rivers or underground water naturally, when mineral deposits or rocks containing arsenic dissolve. Arsenic may also get into water through the discharge of industrial wastes and by the deposit of arsenic particles in dust, or dissolved in rain or snow. Arsenic contamination of ground water became a high-profile problem in recent years due to the use of underground water (tube well water) for drinking purposes, causing serious arsenic poisoning to a large number of people in the world especially in Bangladesh and West Bengal of India (Biswas et al., 1998; Chen et al., 1985; Das et al., 1996; Dhar et al., 1997).

1.5.3 Exposure from food

Recently exposure to arsenic through food has created an attention as high concentrations of arsenic have been found in the different kind of vegetables, dairy products, meats, grains (Al Rmalli et al., 2005; Grotti et al., 2008; Meharg and Rahman, 2003) and other food materials. Food grains and agricultural products have been being cultivated by using groundwater with high concentration of arsenic. Recently high concentration of arsenic was found in the rice of Bangladesh (Al Rmalli et al., 2005; Duxbury et al., 2011; Smith et al., 2006; Williams et al., 2006). Rice is a staple food in many countries including Bangladesh. Several studies have confirmed that contaminated ground water used to cultivate vegetables and rice consumed by people may be an important pathway of ingesting arsenic (Chakraborti et al., 2003). Al Rmalli et al., (2005) have investigated arsenic levels in food imported from Bangladesh to the United Kingdom. Results of this study has suggested that imported vegetables from Bangladesh have 2-100 fold higher concentrations of arsenic than vegetables cultivated in the United Kingdom, European Union, and North America. Further, Huq et al., (2006) conducted a study in Bangladesh with 2,500

water, soil and vegetable samples from arsenic-endemic and non-endemic areas and found that some commonly-grown vegetables, which would usually be suitable as good sources of nourishment, accumulate substantially-elevated amounts of arsenic. Although it has been established that arsenic enters the food chain but there is great uncertainty about the bioavailability and associated toxicity of arsenic from different foods.

1.5.4 Occupational exposure

Exposure to arsenic occurs occupationally in several industries, including mining, wood preservation, pesticide, pharmaceutical, glass, ceramic and microelectronics (IARC, 1980; NRC, 1999). Exposure to arsenic occurs via the oral route (ingestion), inhalation, dermal contact, and the parenteral route to some extent. Inhalation is the principal route of arsenic exposure in occupational settings. Workers who produce or use arsenic compounds in such occupations as vineyards, ceramics, glass-making, smelting, pharmaceuticals, refining of metallic ores, pesticide manufacturing and application, herbicides, fungicides, algaecides, sheep dips, wood preservation, or semiconductor manufacturing may be exposed to substantially higher levels of arsenic (Jones, 2007; Tchounwou et al., 1999). Arsenic is well absorbed by oral and inhalation routes, primarily metabolized in liver and excreted through urine within a few days after consuming any form of inorganic arsenic.

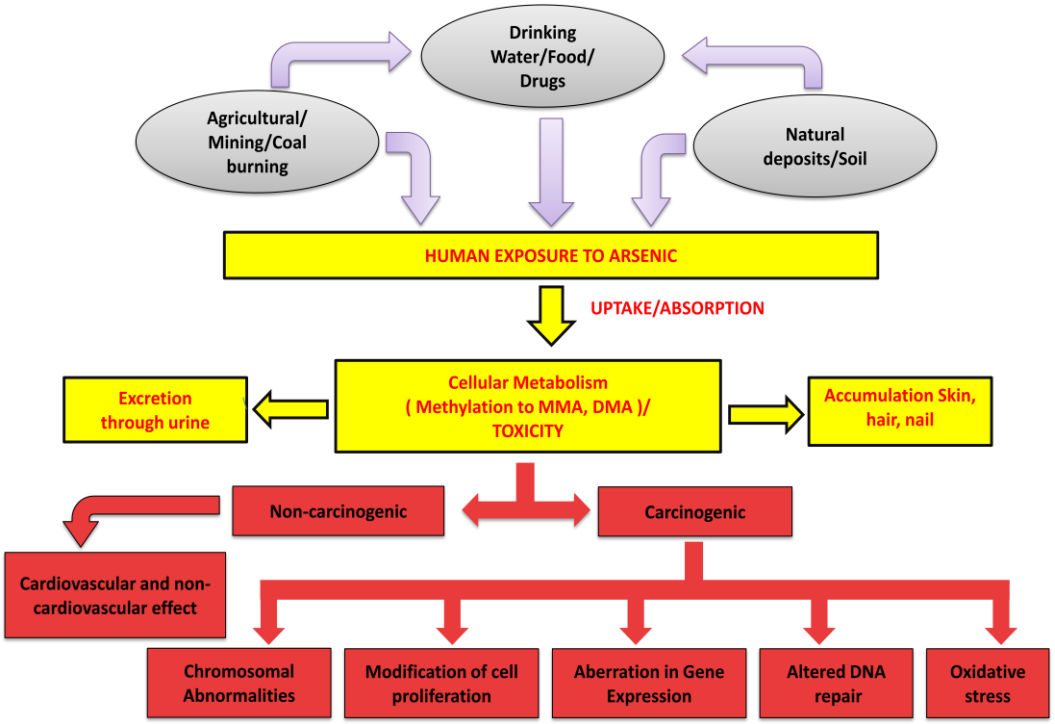

Figure 1.2 Arsenic exposure, metabolism and toxicity in human

Sources: [Roy and Saha, 2002]

1.6 Metabolism of arsenic

The liver is the primary target 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 (Figure 1.2). 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). Inorganic arsenic i.e. arsenic (III) is methylated in 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 (Figure 1.3). 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 less toxic than arsenite or arsenate (Marafante and Vahter, 1987; Vater and Concha, 2001). Inorganic arsenic and its methylated metabolites are mostly excreted through urine within 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 types and patterns of methylated arsenic species in urine are similar 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.

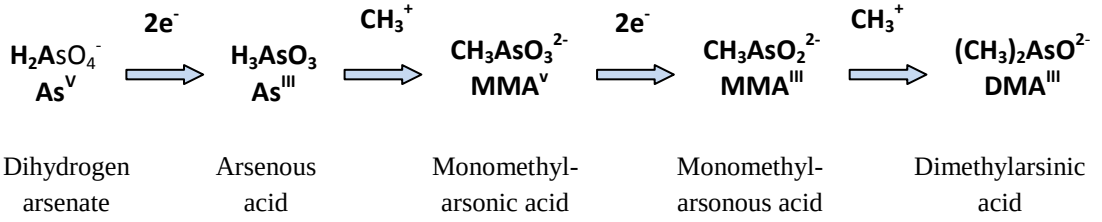

Figure 1.3 Arsenic methylation

Source: [Vater and Concha, 2001]

1.7 Arsenic in different regions of the world

Arsenic toxicity is a global health problem affecting many millions of people. Over the past two or three decades, occurrence of high concentrations of arsenic in drinking-water has been recognized as a major public-health concern in several parts of the world. With the discovery of newer sites in the recent past, the arsenic-contamination scenario around the world, especially in Asian countries, has changed

considerably (Mukherjee et al., 2006). Ground water in different regions of the world is contaminated with arsenic and there are a number of regions including both developing and developed countries where arsenic contamination of drinking water is significant. As a result, arsenic toxicity has created a major public health concern throughout the world. A major and possibly the dominant source of arsenic exposure to human and livestock are drinking of arsenic-contaminated ground water. Millions of people are exposed to elevated levels of toxic inorganic arsenic through the drinking of contaminated ground water and food (Ng et al., 2003; Smith et al., 2000; Meharg, 2008). Most severely affected countries that include Bangladesh, China, Nepal, India, Myanmar, Pakistan, Cambodia, Vietnam, Argentina, Australia, Canada, Greece, Chile, Hungary, Japan, Mexico, Mongolia, New Zealand, South Africa, Philippines, Taiwan, Thailand, USA, etc. where the main source of human exposure is the mass use of arsenic-contaminated ground water for drinking and irrigation for agricultural production (Berg et al., 2007; IARC, 2004; Nicolli et al., 1989; Nordstrom 2002; Razo et al., 1990; Tseng, 1999).

In Asia, arsenic menace is more devastating in some countries specially the arsenic poisoning in alluvial deltas of Gangs River in India and Bangladesh (Dhar et al., 1997; Mandal et al., 1996; Rahman et al., 2001). Sun (2004) reported that the groundwater of the large areas of China and Inner Mongolia has also been contaminated with arsenic resulting in the toxicity of millions of people. Residents in the large alluvial deltas of the Mekong River in southern Vietnam and Cambodia and the Red River in northern Vietnam have been exposed to arsenic mainly through drinking water reported by Berg et al., (2007). Recently, ground water contamination by arsenic has also been reported in Iran (Mosaferi et al., 2008).

In Europe, the arsenic problem is most alarming in Hungary, Serbia and Croatia (Gurzau and Gurzau, 2001; Sancha and Castro, 2001) where in Hungary an inventory of ground water quality conducted (Csalagovits, 1999) which demonstrated that arsenic concentrations in drinking water for 400 towns and villages in the Great Hungarian Plain are several times higher than the guidelines of WHO and European Union (EU).

In the American region, Mexico, United States, Chile and Argentina are the most arsenic affected areas (Nicolli et al., 1989; Razo et al., 1990; Welch et al., 1988) where in some wells in Latin America including Bolivia and Peru, extremely high concentrations of arsenic were found. Different regions of the world having arsenic contaminated ground water are shown in Figure 1.4

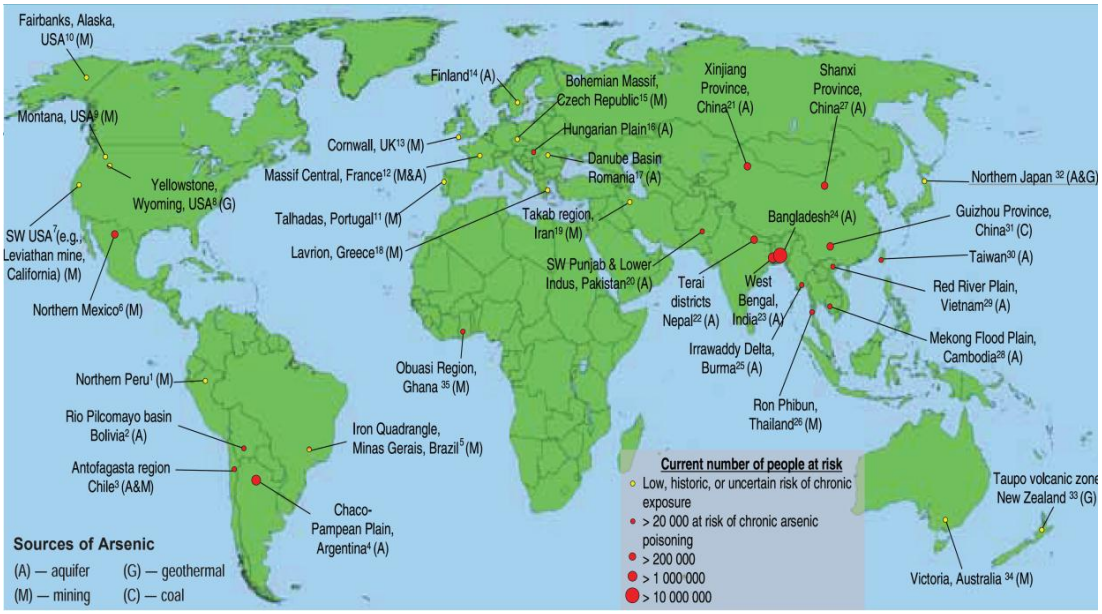

Figure 1.4 Worldwide distributions of arsenic contaminated regions, showing source of arsenic and numbers of people at risk of chronic exposure

Source: [Garelick and Jones, 2008]

1.8 Arsenic contamination in Bangladesh

Bangladesh is a small and densely populated country with an area of 1, 47,570 square kilometers having a population of around 160 millions. This is a common phenomenon here to cope with various natural disasters such as floods, cyclones, tidal bores, and droughts etc. in every year. There is an abundance of surface and ground water because of its flat deltaic land formed by the mechanical action of the great Himalayan Rivers the Ganges, Brahmaputra and Meghna (Safiullah, 2006). Bangladesh is grappling with the largest mass poisoning of a population in history because groundwater used for drinking purposes has been contaminated with naturally occurring inorganic arsenic. According to the Bangladesh Bureau of Statistics, among 160 million inhabitants of Bangladesh 77 million people are affected by arsenic

contaminated water (Flanagan et al., 2012). World Health Organization described the arsenic crisis in Bangladesh as the largest mass poisoning in the human history (Smith et al., 2000;). The scale of this environmental disaster is greater than any seen before; it is beyond the accidents at Bhopal, India, in 1984, and Chernobyl, Ukraine, in 1986.

In 1993, in the Nawabganj district in Bangladesh, naturally-occurring arsenic contaminated water in tube-wells was first confirmed (Khan et al., 1997). As a part of arsenic mitigation programme in Bangladesh, UNICEF tested 4.7 million tube wells for arsenic and 1.4 million of those were found to contain arsenic above the Government drinking water limit of 50 parts per billion (ppb). Combined with another 200,000 unscreened tube wells, which are estimated drinking water to also exceed this limit, it means that almost one in five tube wells is not providing safe drinking water. Nationwide, approximately 20 percent of shallow tube-wells are contaminated. There are more than 8,000 villages where 80 per cent of all tube wells are contaminated. Recent report indicated that about 20 million people in Bangladesh are using tube wells with more than 50 ppb of arsenic (UNICEF, 2009).

Historically, surface water sources in Bangladesh have been contaminated with microorganisms, causing a significant burden of disease and mortality. Infants and children suffered from acute gastrointestinal disease resulting from bacterial contamination of stagnant pond water. Consequently, during the 1970s the United Nations Children's Fund (UNICEF) worked with the Department of Public Health Engineering to install tube-wells to provide what was presumably a safe source of drinking-water for the population. At the time the wells were installed, arsenic was not recognized as a problem in water supplies, and therefore standard water testing procedures did not include tests for arsenic. (UNICEF, 1999). It was considered as a huge success for concern agencies since the primary goal of hand-pumped tube well was achieved. Unfortunately the situation became complex when in 1993, a substantial proportion of the tube wells yielding with high levels of soluble arsenic contaminated water were found (Smith et al., 2000). After then a series of surveys were conducted in between 1995-1998 which revealed that the groundwater of southern and north-eastern Bangladesh has been extensively contaminated with arsenic (BGS 1999; Khan et al., 2003; Smith et al., 2000; Watanabe et al., 2001). In

1998, British Geological Survey (BGS) collected more than 2000 water samples from 41 of the worst-affected districts. This project tested one tube well in every 37 Km^2 in the two third of the country's most affected areas and found that, 51% of the tube wells were contaminated with at least 0.01 mg arsenic/L, 35% with at least 0.05 mg arsenic/L, 25% with at least 0.1 mg arsenic/L, 8% with 0.3 mg arsenic/L or more and 0.1% with 1.0 mg arsenic/L or more (BGS, 1999). In case of arsenic in drinking water, the current WHO recommended guideline is 10µg/L whereas some developing countries including Bangladesh still have a value of 50µg/L (Ng et al., 2003) which is five times higher than the WHO guideline. Already an enormous number of toxicity cases have been reported in the north-west region of Bangladesh and approximately in between 80 to 100 millions of additional people are at risk for arsenic toxicity in the country (Caldwell et al., 2003; Chowdhury 2004). The situation is deteriorating as the new cases of toxicity are still being reported in different parts of the country. There are 61 out of 64 districts (administrative blocks) has been affected by arsenic in Bangladesh (Khan et al., 2006). The entire districts in Bangladesh having arsenic contaminated drinking water exceeding WHO guidelines are shown in Figure 1.5

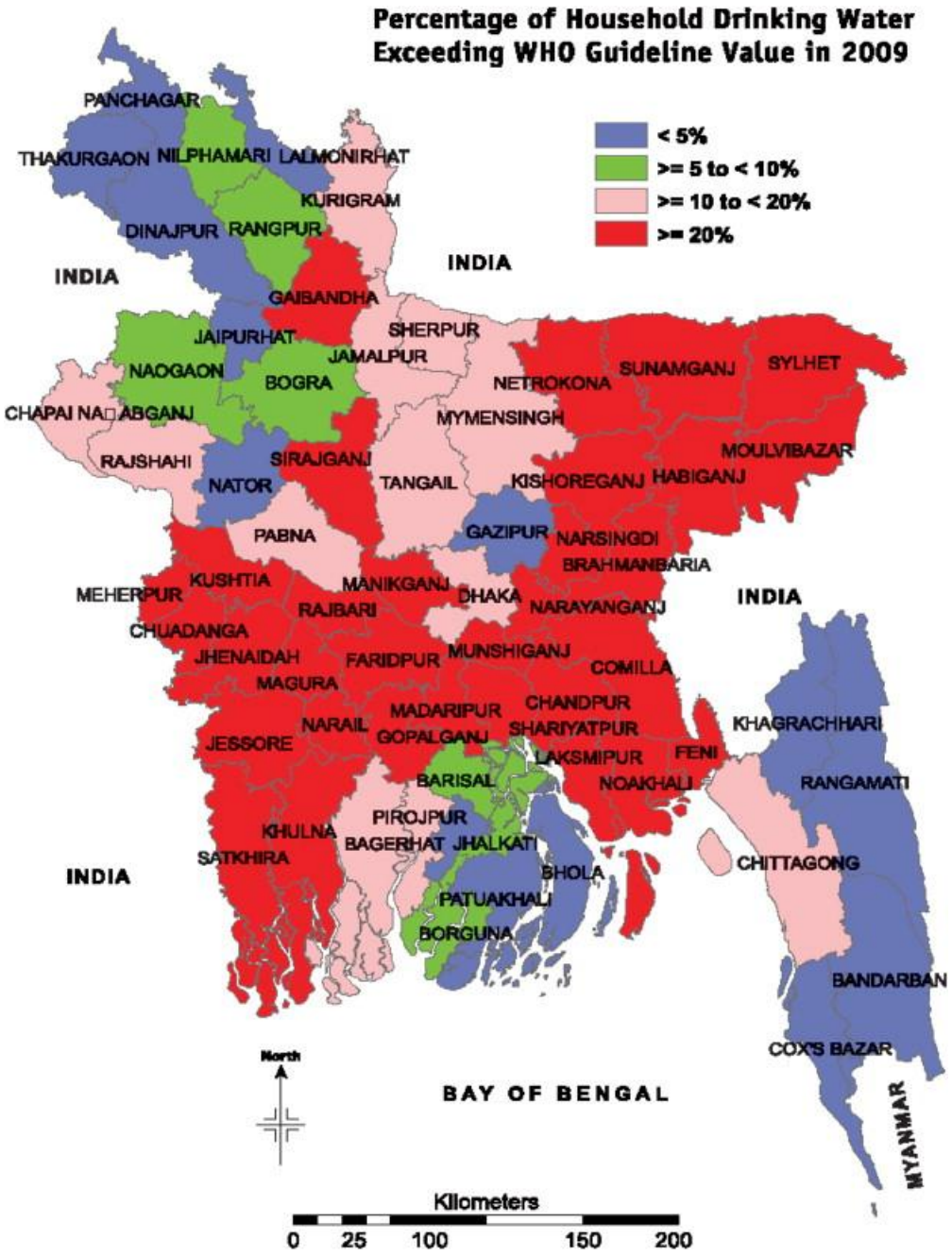

Figure 1.5 Arsenic polluted areas in Bangladesh.

Source: [Executive Summary World Water Day 22 March, 2010 http://www.unicef.org/bangladesh/Towards_an_arsenic_safe_environ_summary%28english%29_22Mar2010.pdf]

1.9 Causes of ground water arsenic contamination in Bangladesh

There is a great temporal and spatial variation of ground water arsenic levels in different regions of Bangladesh. The precise reasons for the high level of arsenic in ground water in Bangladesh is controversial issue and that have not yet been clearly established but several theories have been proposed including role of microbial mobilization, anthropogenic activities, etc. (Harvey et al., 2002; Hossain et al., 2011; Islam et al., 2004; Polizzotto et al., 2006; Sutton et al., 2009). It is now widely believed that the elevated level of arsenic in the ground water in Bangladesh have a natural geological source which may be due to the abstraction of water from quaternary confined and semi-confined alluvial or deltaic aquifers.

There are a huge number of diverse chemical and biological reactions such oxidation, reduction, adsorption, precipitation, methylation and volatilization participate actively in the cycling of arsenicals in the groundwater. A geochemical survey was conducted in six districts of west Bengal bordering the western part of Bangladesh by Das et al. (1996) and the results indicates that the source of arsenic in ground water and soil is the arsenopyrite mineral. However, it is not understood yet clearly about how arsenic is released in ground water from arsenopyrite. In spite of being insoluble in water, pyrite decomposes when exposed to air or aerated water. A feasible explanation for this would be the changes of geochemical environment due to high withdrawal of groundwater resulting decomposition of pyrites to ferrous sulfate, ferric sulfate, and sulfuric acid, thus arsenic in pyrites becomes available (Bridge and Husain 1999; Das et al., 1996; Welch et al., 1988). Due to massive withdrawal of underground water in the last three decades, it may cause the decomposition of pyrites to oxides of iron, arsenic and sulphuric acid which are soluble in water containing sulphuric acid. Under reducing condition below the water table and in the presence of organic matter, non toxic oxides of arsenic are reduced to toxic oxide forms. In 1999, British Geological survey reported that the 'pyrite oxidation' hypothesis proposed by scientists from West Bengal is not a major process for arsenic recruitment in ground water and also stated that ‘oxyhydroxide reduction’ hypothesis proposed by Nickson et al., (1998) is probably the main cause of arsenic mobilization in groundwater. According to this hypothesis, the origin of arsenic rich ground water is due to a

natural process, and it seems that the arsenic in ground water has remained for thousands of years without being flushed from the delta. Arsenic is assumed to be present in alluvial sediments with high concentrations in sand grains as a coating of iron hydroxide. The sediments deposited in valleys eroded in the delta when the stream base level was lowered due to the drop in sea level during the last glacial advance. The organic matter deposited with the sediments reduces the arsenic bearing iron hydroxide and releases arsenic into groundwater. Organic matter deposited in the sediments reduce the arsenic adsorbed on the oxy-hydroxides and releases arsenic into the ground water and dissolution occurs during recharge, caused by microbial oxidation of the organic matter as bacteria dissolves surrounding oxygen.

1.10 Social stigma of arsenic toxicity in Bangladesh

In Bangladesh, people with arsenic poisoning suffer enormous social stigma. Many people believe, arsenic poisoning is infectious or a curse. Usually parents are reluctant to let their children play with other children suffering from arsenic poisoning and patients of arsenic poisoning can be shunned within their villages. In case of women, the situation is more serious. Usually in Bangladesh, a women's magnetism lies in her beauty which is judged by her pale complexion. Unfortunately, this makes it harder and in some cases impossible to get marry, for single woman who is suffering from arsenic-induced melanosis and keratosis of the skin. Even once married, after then women have to face the risk of divorce if they develop arsenic exposure-related skin diseases. In fact, this is a big social problem in male-dominated society in Bangladesh. (UNICEF, 2009).

1.11 Standards and regulations for arsenic exposure through drinking water

According to the World Health Organization (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. Efforts of WHO to reduce arsenic exposure 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 (GDWQ) .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 current recommended limit of arsenic in drinking water is 10 μg/L although this guideline value is designated as provisional due to the measurement difficulties and practical difficulties in removing arsenic from drinking-water. This is based on a 6×10^{-4} excess skin cancer risk, which is 60 times higher than the factor normally used to protect human health. However, the WHO states that the health-based drinking water guideline for arsenic should in reality be 0.17 μg/L (Kapaj et al., 2006)

The US EPA drinking water standard for arsenic was set at 50 μg/L in 1975, based on a Public Health Service standard originally established in 1942 (US EPA) Recently, the US EPA has established a health based, non-enforceable Maximum Contaminant Level Goal (MCLG) of zero arsenic and an enforceable Maximum Contaminant Level (MCL) of 10 μg As/L in drinking water (US EPA). However, the current drinking water guideline for arsenic adopted by both the WHO and the US EPA is 10 μg/L. This is higher than the proposed Canadian and Australian maximum permissible concentrations of 5 and 7 μg As/L, respectively. (Kapaj et al., 2006)

1.12 Minimal Risk Levels (MRLs) for arsenic

An MRL is an estimate of the daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse non-cancer health effects over a specified duration of exposure. They are below levels that might cause adverse health effects in the people most sensitive to such chemical-induced effects. MRLs are derived for acute (1-14 days), intermediate (15-364 days), and chronic (365 days and longer) durations and for the oral and inhalation routes of exposure (http://www.opentoxipedia.org/). Health effects of arsenic are determined by the dose, the duration (how long), and the route of exposure. According to Agency for Toxic Substances and Disease Registry (ATSDR) acute-, intermediate- or chronic-duration inhalation MRLs were not derived for any inorganic arsenic or organic arsenic compounds. For inorganic arsenic an MRL of 0.005 mg arsenic/kg/day has been

derived for acute-duration oral exposure (≤ 14 days) but there is no intermediate-duration oral MRL was derived for inorganic arsenic. For chronic-duration oral exposure (≥ 1 year) to inorganic arsenic an MRL of 0.0003 mg arsenic/kg/day has been derived. There is no acute-duration oral MRL derived for monomethylarsonic acid (MMA). An MRL of 0.1 mg MMA/kg/day has been derived for intermediate-duration oral exposure (15-364 days) to MMA and for chronic-duration oral exposure (≥ 1 year) the MRL is 0.01 mg MMA/kg/day. For DMA there is no acute- or intermediate duration oral MRLs but an MRL of 0.02 mg DMA/kg/day has been derived for chronic-duration oral exposure (≥ 1 year) to DMA. The normal human levels of arsenic in unexposed individuals are < 1 µg/L in blood, <100 µg/L in urine, ≤1 ppm in nails and ≤1 ppm in hair (ATSDR, 2007)

1.13 Health effects of arsenic

Worldwide chronic arsenic toxicity has become a major threat to human health. Arsenic exposure to humans mainly occurs from the ingestion of arsenic contaminated water and food. Chronic exposure to arsenic has more effects on health than any other toxicant, and the list continues to grow. Arsenic poisoning takes between 8 and 14 years to its effect on health, depending on the amount of arsenic ingested, nutritional status, and immune response of the affected individual (Ahsan et al., 2006; Maharjan et al., 2007; Milton et al., 2004; Watanabe et al., 2001). Several studies have clearly indicated that the toxicity of arsenic depends on the exposure dose, frequency and duration, age, and sex, as well as on individual susceptibilities, genetic and nutritional factors (Ahsan et al., 2007). Toxicity of arsenic has been heightened by recent reports of large populations in Bangladesh, West Bengal, Inner Mongolia, Taiwan, China, Mexico, Argentina, Chile and Hungary that have been exposed to high concentrations of arsenic in their drinking water (Argos et al., 2010; Chen et al., 1988a, 1988b; Mandal and Suzuki, 2002; Meliker et al., 2007; Mukherjee et al., 2006).

Human health effects of chronic arsenic toxicity (CAT) are designated by the term arsenicosis which was first coined by Guha Mazumder et al. (1988) group and later used by WHO to imply a chronic disease caused by prolonged exposure in humans to arsenic. Previously the condition were described as arseniasis, arsenism,

arsenicism, etc (Guha Mazumder, 2008). Many different systems or organs within the body are affected by chronic exposure to inorganic arsenic, particularly because of its potential to be a human carcinogen. The clinical manifestations of arsenic toxicity are many, but the most commonly observed symptoms in people who suffer from chronic arsenic poisoning are the characteristic skin lesions. The main dermatological symptoms observed in arsenic affected people are melanosis (change of pigmentation) and keratosis (rough, dry, papular skinlesions). Chronic arsenic exposure may also cause reproductive, neurological, cardiovascular, respiratory, hepatic, hematological, and diabetic effects in humans (NRC, 1999). Intake of inorganic arsenic was recognized as a cause of skin, bladder, and lung cancer (Cantor, 2001; IARC 2004). A good number of articles have been published on chronic arsenic exposure and its associated health effects (Chen et al., 2007; Hossain et al., 2012; Huda et al., 2014; Islam et al., 2011; Kapaj et al., 2006; Mandal and Suzuki 2002; Morton and Dunnette, 1994; Naqvi et al., 1994; Ng et al., 2003; Wang et al., 2007; Yoshida et al., 2004)

1.13.1 Skin manifestations

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). Recently, 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 1.6)

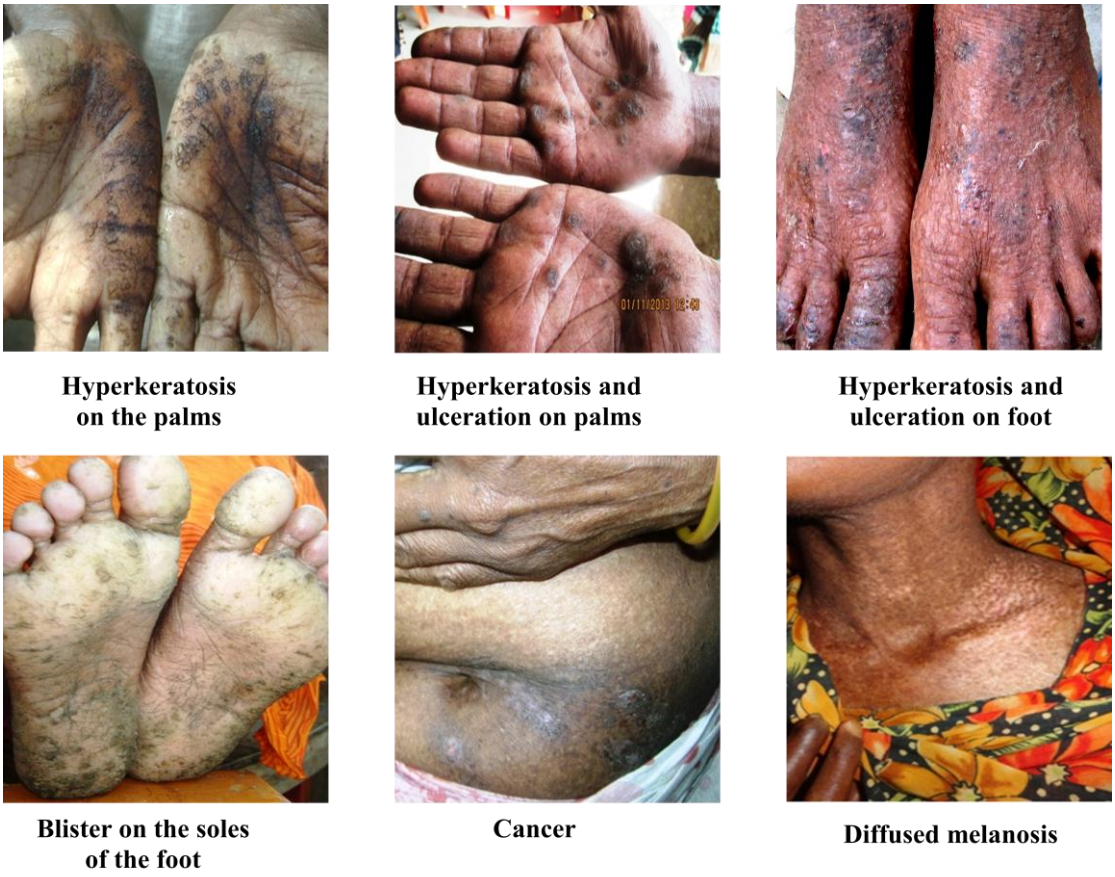

Figure 1.6 Some phenomenon caused by chronic exposure to arsenic

Source: [Environmental Health Sciences group, Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi, Bangladesh]

1.13.2 Carcinogenic effects

The evidence of carcinogenicity in humans from exposure to arsenic is based on epidemiological studies of cancer in relation to arsenic in drinking water. The Working group of International Agency for Research on Cancer (IARC) and the US Environmental Protection Agency (EPA) evaluated data from ecological studies, cohort studies and case-control studies from many countries and observed that arsenic was potentially carcinogenic for skin cancer (IARC, 2004; Smith et al., 1992). The carcinogenic potential of arsenic was recognized over 110 years ago by Hutchison, who observed an unusual number of skin cancer occurring in patients treated for various diseases with medical arsenicals (Klassen, 2008).

Different epidemiological studies demonstrated an evident causal relationship between environmental, occupational, and medical exposure of millions of people worldwide 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). It is thought that the mechanism by which these cancers originate may involve the promotion of oxidative stress by arsenic compounds, in which the antioxidant capacity of the living organism is overwhelmed by ROS (reactive oxygen species), resulting in molecular damage to proteins, lipids and most significantly DNA (Cohen et al., 2006; Lynn et al., 2000; Shi et al., 2004; Valko et al., 2005).

In exposed human populations, arsenic has been primarily associated with tumors of the skin and lungs, but also can be associated with tumors of the bladder, kidney, and liver. A large number of epidemiological studies have reported that inhalation exposure to inorganic arsenic increases the risk of lung cancer (Smith et al., 1998; Enterline et al., 1995; Järup et al., 1989). Rossman et al. (2004), pointed out that arsenic can play a role in the enhancement of UV-induced skin cancers. The mechanism of action may involve effects on DNA methylation and DNA repair. Malignant arsenical skin lesions may be Bowen's disease (intraepithelial carcinoma, or carcinoma *in situ*), and multiple basal cell carcinomas, arising from cells not associated with hyperkeratinization or squamous cell carcinomas, which develop from some of the hyperkeratotic arts or corns. Skin cancer may arise in the hyperkeratotic areas or may appear on non-keratotic areas of the trunk, extremities, or hand. Arsenic in drinking water is associated with kidney cancer. Ecological studies in Taiwan, Chile, Argentina and Australia, and cohort studies from Taiwan and the USA demonstrated that long-term exposure to arsenic increased the risks for kidney cancer (Chen et al., 1985, 1988a, 1992; Hopenhayn-Rich et al., 1998; Kurttio et al., 1999). There is general agreement that inhalation of inorganic arsenic has been documented as a lung carcinogen in humans. Lung cancer is the leading cause of cancer-related mortality in the United States and worldwide. 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 cancers can develop from specific chronic liver diseases. Liver cirrhosis appears to be a primary cause of arsenic-related mortality in Guizhou, China, and is potentially associated with hepatocellular carcinoma (Liu et al., 1992; Liu et al., 2002). International Agency for Research on Cancer listed the liver as a potential organ for arsenic carcinogenesis (IARC, 2004). The association between environmental arsenic exposure and human liver cancers has been repeatedly reported (Centeno et al., 2002; Chiu et al., 2004; Liaw et al., 2008). Although it is clear that arsenic is a human carcinogen, the precise cellular mechanism by which arsenic induces cancer is still largely unknown.

1.13.3 Cardiovascular effects

Cardiovascular disease is the most common cause of death worldwide. Atherosclerosis is the central event of cardiovascular disease and proatherogenic role of arsenic have been well established (Chen et al., 1988b; Chiou et al., 1997; Hossain et al., 2012; Mumford et al., 2007; Rahman et al., 1999; Sohel et al., 2009; Tseng et al., 2003, Tseng, 2005; Wang et al., 2002). The cardiovascular system is a very sensitive target of arsenic toxicity. Arsenic-induced cardiovascular diseases in human population 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; Huda et al., 2014; Karim et al., 2013; Mumford et al., 2007; Rahman et al., 1999; Sohel et al., 2009; Tseng et al., 2003, 2005; Wang et al., 2002). The first evidence of a link between cardiovascular disease 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 cardiovascular disease was reported in smelter workers due to arsenic exposure (Axelson et al., 1978; Lee-Feldstein 1989). Recently we found that arsenic exposure causes endothelial damage or dysfunction, an early event of atherosclerosis (Hossain et al., 2012).

It is believed that vascular endothelial cells play a pivotal role in arsenic-induced cardiovascular diseases. Arsenic causes endothelial damage through reactive oxygen species (ROS) production. Endothelial cell activation/dysfunction by arsenic increases the production of several inflammatory and adhesion molecules such as soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), monocyte chemotractant protein-1 (MCP-1) related to the atherosclerotic lesions (Blankenberg et al., 2001; Chen et al., 2007; Hsieh et al., 2008; Hwang et al., 1997; Lee et al., 2005; Ridker et al., 1998).

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 cardiovascular diseases (Axelson et al., 1978; Lee et al., 2002; Lee-Feldstein, 1983; Wu et al., 1989). A dose-response relationship between prevalence of CVD and ingested arsenic was reported in northeastern Taiwan (Chiou et al., 1997). Ischemia is localized tissue anemia 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, (BFD) a unique form of peripheral vascular disease, has been reported to be one of the important complication of chronic arsenic toxicity in southwestern 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. Increased prevalence of peripheral vascular disease has also been reported among residents with long-term arsenic exposure from arsenic present in drinking water in Taiwan, Chile, the USA, and Mexico (Chen et al., 2007; NRC 1999, 2001; Tseng et al., 1996; Wang et al., 2007).

1.13.4 Hepatotoxic effects

Liver is the major site of arsenic detoxification. 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 in the drinking water (Mazumder, 2005; Islam et al., 2011), or from environmental exposure to arsenic through burning high-arsenic coal in interior stoves (Liu et al., 1992). Histological examination of the livers has revealed a

consistent finding of portal tract fibrosis (Mazumder, 2005). Individuals 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 highly contaminated with 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 prolong drinking of arsenic-contaminated water is associated with hepatomegaly, hepatic fibrosis and cirrhosis.

1.13.5 Renal effects

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. Sites of arsenic damage in the kidney include capillaries, tubules, and glomeruli (Winship, 1984). Damaged proximal tubular cells lead to proteinuria and casts in the urine. In some cases elevated levels of creatinine or bilirubin have been reported (Moore et al., 1994). Mitochondrial damage is also prominent in tubular cells. Several animal studies have reported renal effects following intermediate- or chronic-duration of oral arsenic exposure (Brown et al., 1976). The effects include increased kidney weight, swollen mitochondria and increased numbers of dense autophagic lysosome-like bodies in the proximal tubules, increased pigmentation in the proximal tubules, and cysts.

1.13.6 Neurological effects

The most typical neurological feature of arsenic neurotoxicity is peripheral neuropathy (Mathew et al., 2010). A large number of epidemiological studies and case reports in arsenic affected areas revealed that chronic arsenic exposures are associated with various neurologic problems such as mental retardation, and developmental disabilities such as physical, cognitive, psychological, sensory and speech impairments (Saha, 1995; Winship, 1984; Zierold et al., 2004). Studies on patients with arsenic neuropathy have shown a reduced nerve conducting velocity in their peripheral nerves, and this has become a hallmark of arsenic-induced

neurotoxicity. Studies in China and Bangladesh have shown that mental health problems (e.g. depression) are more common among the people affected by arsenic contamination (Brinkel et al., 2009). Additionally, a significant association between decreased reading and spelling performance and hair arsenic levels was found in a group of elementary school children (Moon et al., 1985), suggesting that arsenic may also cause neurobehavioral effects. Like the cardiovascular system, both the peripheral and central components of the nervous system can be damaged by arsenic (Saha 1995; Winship 1984). Symptoms of chronic encephalopathy include persistent headache, diminished recent memory, distractibility, abnormal irritability, restless sleep, loss of libido, increased urinary urgency, and increased effects of small amount of ethanol (Morton and Caron, 1989). Secondary depression, anxiety, panic attacks and somatizations are common. In addition, experiences with animals have pointed out that prenatal arsenic exposure was associated with depressive-like behaviors in the affected offspring mouse (Martinez et al., 2008).

1.13.7 Reproductive effects

Arsenic exposure has been associated with a number of adverse health outcomes, but relatively little attention has been directed toward the potential impact of arsenic on human reproductive system. Both animals and human experiments have demonstrated that arsenic and its methylated metabolites cross the placenta (Concha et al., 1998; He et al., 2007), and thus fetuses may be exposed to arsenic. Several studies suggest the association between arsenic exposure and adverse pregnancy outcomes, such as spontaneous abortion and stillbirth, and infant death (Ahmad et al., 2001; Milton et al., 2005; Rahman et al., 2007; von Ehrenstein et al., 2006). In a study with mice, a significant decrease in sperm count and motility along with increase in abnormal sperm were observed at high concentration (Pant et al., 2001).

1.13.8 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, and 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 free radical 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).

1.13.9 Respiratory effects

Effects of arsenic on the human respiratory system have been reported from both occupational exposure as well as from tube-well water arsenic toxicity. Human exposed to arsenic dust or fume inhalation are more opt to be encountered in mining and milling of ores, in industrial processing, such as smelting industry which often produces irritation of the mucous membrane, resulting in laryngitis, bronchitis, rhinitis and tracheobronchitis, causing stuffy nose, sore throat, hoarseness and chronic cough etc. (Dekundt et al., 1986; Saha, 1999). A fatal case of arsenic trioxide inhalation manifested widespread as tracheobronchial mucosal and sub mucosal hemorrhages with mucosal sloughing, alveolar hemorrhages, and pulmonary edema (Gerhardsson et al., 1988).

Noncancerous respiratory effects of arsenic on the human have been reported both from occupational exposure as well as from tube well water arsenic toxicity. Mazumder et al., (2000) reported an association between arsenic ingestion in drinking water and the prevalence of respiratory disorders. The relationship between ingested arsenic and nonmalignant respiratory effects has so far only been reported from Chile, India, and Bangladesh (Smith, 1998). Ingestion of inorganic arsenic for a prolonged period causes respiratory problems, including cough, chest sound, bronchitis, and shortness of breath (Mazumder et al. 2000; Milton and Rahman, 2002; Milton et al., 2001, 2003; Islam et al. 2007). In a small cross-sectional study, Milton and Rahman, (2002) investigated the link between arsenic exposure and the rate of chronic bronchitis in Bangladesh and they concluded that ingestion of inorganic arsenic present in drinking water may lead to increased risk of chronic cough and bronchitis. Saha et al. (1995) conducted a study in West Bengal of India, and found a good number of patients with asthmatic symptoms in arsenic-endemic areas. They concluded that bronchitis and asthma were the common complications of ground water arsenic toxicity. Recently Islam et al. (2007) studied the link between arsenic exposure via drinking water and respiratory complications. They found high prevalence of respiratory complications such as breathing problems including chest sound, asthma, bronchitis, and cough in arsenicosis patients in Bangladesh. Asthma is a chronic disease worldwide. According to Global Initiative for Asthma (GINA)

estimates, 300 million people worldwide currently suffer from asthma (Masoli et al. 2004). Asthma is the most common chronic disease among children and adults. Asthma is a major public health concern in both developing and developed countries (Braman, 2006; Masoli et al. 2004). Mortality rate caused by asthma is still high. Although associations of arsenic exposure with other diseases have been well documented, a very little attention has been given on arsenic exposure-related asthma.

1.13.9.1 Asthma

1.13.9.1.1 Definitions of asthma

There is no clear, universally acceptable definition of asthma because of the absence of any gold standard for asthma (Torén et al., 1993). Definitions have been offered by a variety of expert groups (Table 1.2), but none is sufficiently precise to allow asthmatic subjects to be distinguished from non-asthmatic subjects, based on the definition alone. All definitions have their central criteria as a physiologic phenomenon: reversible bronchospasm or increased airway responsiveness (Weiss, 1990)

Table 1.2 Definitions of asthma

National Heart Lung and Blood Institute (National Asthma Education and Prevention Program, 2007)	Asthma is a common chronic disorder of the airways that is complex and characterized by variable and recurring symptoms, airflow obstruction, bronchial hyper-responsiveness, and an underlying inflammation
Ciba Foundation Guest Symposium, 1959	Asthma refers to the condition of subjects with widespread narrowing of the bronchial airways, which changes its severity over short periods of time either spontaneously or under treatment.
American Thoracic Society (ATS, 1962)	Asthma is a disease characterized by an increased responsiveness of the trachea and bronchi to various stimuli and manifested by a widespread narrowing of the airways that changes in severity either spontaneously or as a result of therapy.

American College of Chest Physicians (Chest 1975)	Asthma is a disease characterized by an increased responsiveness of the airways to various stimuli and manifested by slowing of forced expiration which changes in severity either spontaneously or as a result of therapy.
World Health Organization (WHO 1975)	Asthma is a chronic condition characterized by recurrent bronchospasm resulting from a tendency to develop reversible narrowing of the airway lumina in response to stimuli of a level or intensity not inducing such narrowing in most individuals.

1.13.9.1.2 Global prevalence of asthma

There has been a sharp increase in the global prevalence, morbidity, mortality, and economic burden associated with asthma over the last 40 years. Approximately 300 million people worldwide currently have asthma, and its prevalence increases by 50% every decade (Masoli et al. 2004; Braman, 2006). Prevalences are high (>10%) in developed countries and rates are increasing in developing regions as they become more westernized. The most striking increases are seen among children although the disease is also on the increase in the elderly (Braman, 2003; Braman, 2006). Worldwide, approximately 180,000 deaths annually are attributable to asthma (Braman, 2003). Figure 1.7 shows the global prevalence of clinical asthma.

1.13.9.1.2.1 Prevalence in developed countries

A broad consensus exists that in most Western countries the prevalence of asthma increased over the last four decades of the 20th century.(Anderson et al., 2004) The highest asthma prevalence are found in the United Kingdom (>15%), New Zealand (15.1%), Australia (14.7%), the Republic of Ireland (14.6%), Canada (14.1%), and the United States (10.9%) (Masoli et al. 2004). One in every 10 persons has asthma in North America that accounts for approximately 35.5 million asthma patients in this region. Certain ethnic groups, such as African Americans and Hispanics, have an even higher prevalence. In Western Europe, almost 30 million individuals now have asthma and the prevalence rate has doubled over the last decade.

(European lung white book, 2003). In the United Kingdom, one estimate suggests that 3.4 million people, namely 1 in every 7 children aged 2 to 15 years (1.5 million) and 1 in every 25 adults (1.9 million), have asthma symptoms requiring treatment (European lung white book, 2003)

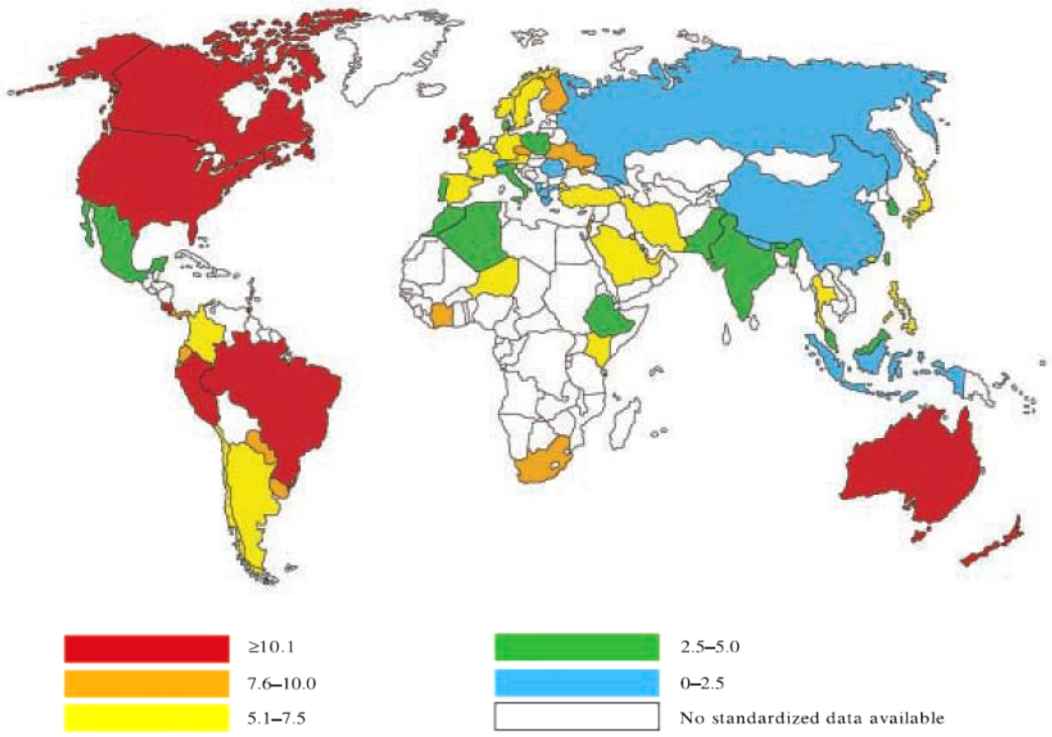

Figure 1.7 World-wide prevalence of asthma.

Source: [Masoli et al., 2004]

1.13.9.1.2.2 Prevalence in developing countries

In developing regions (Africa, Central and South America, Asia, and the Pacific), asthma prevalence continues to rise sharply with increasing urbanization and westernization (Masoli et al., 2004). Estimates suggest that >40 million individuals in South and Central America and > 50 million individuals in Africa currently have the disease. High prevalences have been reported in Peru (13.0%), Costa Rica (11.9%), and Brazil (11.4%) (Masoli et al., 2004). In Africa, asthma prevalence is highest in South Africa (8.1%), perhaps the most westernized of the African countries (Masoli et al., 2004). Almost 44 million people in the East Asia/Pacific region have asthma, although the prevalence rates vary markedly throughout the region. In Asia, increased

prevalences are likely to be particularly dramatic in India and China. For example, a 2% increase in prevalence in China would lead to an additional 2 million asthma sufferers. In India the overall prevalence of asthma is 2.38% (Aggarwal et al., 2005). In Bangladesh asthma appears to be a substantial public health problem. In an estimate it has shown that 7 million people including 4 million children in Bangladesh suffering from asthma related symptoms and the prevalence of asthma is 6.9% (Hassan et al., 2002).

1.13.9.1.3 Pathophysiology of asthma

Asthma is a chronic inflammatory disorder of the airways. The chronic inflammation leads to recurrent episodes of wheezing, breathlessness, chest tightness, and cough, particularly at night or in the early morning. These episodes are associated with widespread but variable airflow obstruction that is usually reversible, either spontaneously or with treatment. The clinical course of asthma is unpredictable, ranging from periods of adequate control to exacerbations with poor control of symptoms. (National Asthma Education and Prevention Program, 2007). In the inflammatory process of asthma, many inflammatory cells participate and mediate a complex mixture of mediators. Cytokines are of particular importance as mediators of chronic inflammation and the means by which cytokines amplify and perpetuate the inflammatory process is now emerging. Airway epithelial cells may be a particularly important source of cytokines and other mediators. Several inflammatory processes have been reported to be involved in bronchial constriction leading to asthma. However asthma results not only from bronchoconstriction, but also includes plasma exudation, the activation of neural mechanisms, mucus secretion. The chronic inflammation may lead to structural changes, including an increase in airway smooth muscle and fibrosis, that are essentially irreversible (Figure 1.8) (Barnes, 1996).

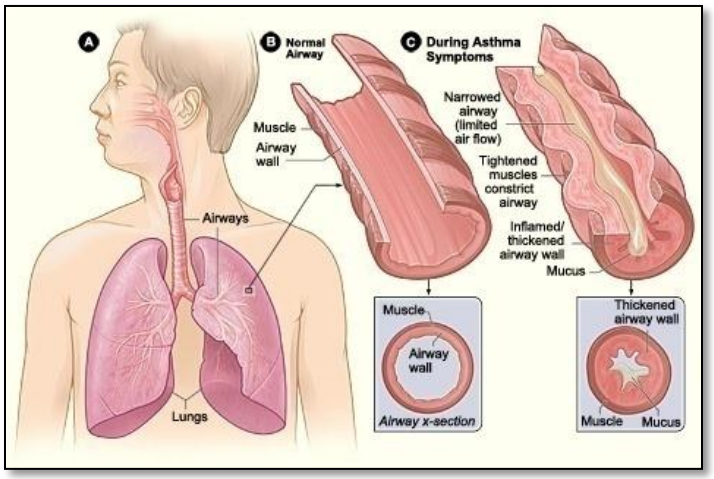

Figure 1.8 Pathophysiology of asthma. (A) shows the location of the lungs and airways in the body. (B) shows a cross-section of a normal airway. (C) shows a cross-section of an airway during asthma symptoms

Source: [NIH, National Heart Lung and Blood Institute http://www.nhlbi.nih.gov/health/health-topics/topics/asthma]

1.13.9.1.4 Circulating molecules related to asthma

1.13.9.1.4 .1 Immunoglobulin E

It has been reported that that several soluble molecules and factors have been implicated in asthma. Allergy is a major risk factor for developing asthma (Holt et al., 1999). Immunoglobulin E, a main mediator of allergic reaction has been implicated in bronchial asthma (Burrows et al.,1989; Sporik et al., 1990). A hypersensitivity reaction mediated by IgE antibodies induces allergic asthma. IgE plays a central role in the initiation and propagation of the inflammatory cascade and thus the allergic respons (Sandeep et al., 2010)**.** The evidence for a causal relationship between allergens and asthma hinges on epidemiologic findings showing a strong association between specific IgE antibodies, or total IgE and asthma (Burrows et al., 1989; Sporik et al., 1990; Sears et al., 1991; Sunyer et al., 1995). In an epidemiological study, Burrows and colleagues (1989) found a close correlation between serum IgE levels and self-reported asthma. In the study, they concluded that "asthma is almost always associated with some type of IgE-related reaction and therefore has an allergic basis".

1.13.9.1.4 .2 Cytokines

Cytokines are usually extracellular signalling proteins, usually less than 80 kD in size, and many are glycosylated. They are produced by many different cell types that are involved in cell-to-cell interactions acting through specific receptors on the surface of target cells. They act on target cells to cause a wide array of cellular functions including activation, proliferation, chemotaxis, immunomodulation, release of other cytokines or mediators, growth and cell differentiation, and apoptosis. Cytokines play an integral role in the coordination and persistence of the inflammatory process in the chronic inflammation of the airways in asthma since they are capable of inducing many of the pro-inflammatory effects characteristic of this disease. It is not simple to classify the numerous cytokines that are potentially involved in asthma because of their pleiotropic nature and overlapping properties. (Chung and Barnes, 1999). However, with regard to the specific abnormalities of asthma and to the current understanding of the pathogenesis of asthma, they may be grouped as follows:

(1) Lymphokines: Interleukin-4 (IL-4), IL-5, IL-2, IL-3, IL-13, IL-15, IL-16, IL-17.

(2) Pro-inflammatory cytokines: IL-1, TNF, IL-6, IL-11, GM-CSF, SCF.

(3) Anti-inflammatory cytokines: IL-10, IL- 1ra, IFN-γ, IL-12, IL-18.

(4) Chemotactic cytokines (chemokines): RANTES, MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, MIP-1α, eotaxin, IL-8.

(5) Growth factors: PDGF, TGF-β, FGF, EGF, IGF.

Some cytokines especially IL-4 has a pivotal role in an allergen-mediated IgE production through the classswitching of B lymphocyte into IgE producing B cell (Coffman and Carty,1986; Coffman et al., 1986; Finkelman et al., 1986; Snapper et. Al., 1988). Elevated levels of IgE produced by B cells can cause the activation of mast cells through receptor cross liking. Activated mast cell releases histamin, several cytokines, lipid mediator, prostaglandins that might be involved in the bronchial constriction leading to asthma (Bloemen et al., 2007; Marshall and Jawdat, 2004; Wedemeyer et al., 2000).

1.14 Dissertation aims

Arsenic, a ubiquitous naturally occurring metalloid is considered as one of the most toxic element of the geosphere (Mandal and Suzuki, 2002). Arsenic toxicity in human is mostly associated with the contamination of drinking water from natural geological sources (Matschullat, 2000). Arsenic toxicity is a global health problem affecting many millions of people. Over the past two or three decades, arsenic pollution has been recognized as an important environmental issue and a major public-health concern in several parts of the world. With the discovery of newer sites in the recent past, the arsenic-contamination scenario around the world, especially in Asian countries, has changed considerably (Mukherjee et al., 2006). In Asia, arsenic menace is more devastating in some countries specially the alluvial deltas of Gangs River in India and Bangladesh (Dhar et al., 1997; Mandal et al., 1996; Rahman et al., 2001). Bangladesh has been grappling with the largest mass poisoning of a population in history because of the contamination of drinking water by inorganic arsenic greater than the permissive limit (10µg/L) for the arsenic-endemic people set by World Health Organization (WHO) (Caldwell et al., 2003; Chowdhury, 2004; Khan et al., 1997). Elevated levels of arsenic have been reported in 61 out of 64 districts (administrative blocks) and the scale of disaster has exceeded the Chernobyl catastrophe in Ukraine and Bhopal accident in India (Smith et al., 2000). Chronic arsenic exposure has been reported to be associated with increased risk of a wide range of health outcomes including cancers of the skin, lung, bladder, liver, and kidney, neurologic diseases, cardiovascular diseases and other non-malignant diseases (Ali et al., 2010; Brouwer et al., 1992; Chen et al., 2007; Hopenhayn-Rich et al., 1998; Huda et al., 2014; Karim et al., 2013; Mazumder et al., 1998, 2000; Mazumder, 2005; Meliker et al., 2007; Mumford et al., 2007; Rahman et al., 1999; Smith et al., 1998; Tapio and Grosche, 2006; Tseng, 1977; Vahidnia et al., 2008; Wang et al., 2002; Wu et al., 1989). Several epidemiological studies have suggested that ingestion of arsenic via drinking water for a prolonged period is associated with respiratory complications including asthma (Islam et al., 2007; Mazumder et al., 2000; Saha, 1995, 2003; Saha et. al., 1999). Asthma is an inflammatory disease of the airways of the lung, characterized by intermittent airway narrowing and variable symptoms of chest tightness, wheeze and shortness of breath (National Asthma Education and

Prevention Program, 2007). Asthma is a substantial public health problem among children and adults worldwide and there has been a sharp increase in the global prevalence, morbidity, mortality, and economic burden associated with the disease. Global Initiative for Asthma (GINA) estimates that approximately 300 million people worldwide currently have asthma, and its prevalence increases by 50% every decade (Braman, 2006; Masoli et al. 2004). Worldwide, approximately 180,000 deaths annually are attributable to asthma (Braman, 2003). In an estimate it has shown that 7 million people including 4 million children in Bangladesh suffering from asthma (Hassan et al., 2002). Several genetic and non-genetic factors are responsible for the development of asthma (Sengler et al., 2002; Martinez, 1997). The disease is largely mediated by allergic reactions involving immunoglobulin E (IgE), interleukins, chemokines, adhesion and inflammatory molecules (Barnes, 2008; Burrows et al., 1989; Chung and Barnes, 1999). Interleukin-4 (IL-4) is an important cytokine for IgE-mediated allergic reactions leading to bronchial asthma. Increase production of IgE is a hallmark of allergic asthma. Allergic asthma is the most common form of asthma (NCEH, CDC, 1999). IgE production is regulated by IL-4. IL-4, a cytokine secreted mainly from T_H2 subset of $CD4^+$ helper T cell has been reported to be involved in IgE production through the class switching of B lymphocytes to IgE producing B cells (Abbas et al., 1996; Poulsen and Hummelshoj, 2007). IL-4 is also implicated in the production of other cytokines and mediators involved in the inflammatory process of bronchial asthma. Although arsenic exposure has been reported to be associated with asthma, however, the effects of arsenic exposure on IgE and IL-4 are largely unknown. Therefore, this study was designed to explore the associations of arsenic exposure with circulating IgE and IL-4 levels recruiting human individuals from arsenic-endemic and non-endemic areas in Bangladesh.

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Chapter 2
CHEMICALS AND EQUIPMENTS

CHAPTER 2: CHEMICALS AND EQUIPMENTS

2.1 List of chemicals and test kits

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

1. Human IgE (Immunoglobulin E) ELISA kit
   DRG International, Inc., USA
2. Human IL-4 ELISA Kit
   R&D Systems, Inc., USA
3. Nitric acid
   Merck, Germany
4. Sulphuric acid
   Merck, Germany
5. Hydrochloric acid
   Merck, Germany
6. Triton X-100
   Sigma-Aldrich, Germany
7. Ethanol
   Sigma-Aldrich, Germany
8. Acetone
   Merck, Germany
9. HPLC grade water
   Active Fine Chemicals Ltd., Bangladesh
10. River water as a certified reference material (CRM) for ICP-MS
    NMIJ CRM 7202-a No.347, National Institute of Advanced Industrial Science and Technology, Japan
11. Human hair as a certified reference material (CRM) for ICP-MS analysis.
    GBW09101, Shanghai Institute of Nuclear Research Academia Sinica, China

2. 2 List of equipments

The important equipments used throughout this study are listed below

1. Inductively coupled plasma mass spectroscopy (ICP-MS)
   Model No. HP-4500, Agilent Technologies, Japan
2. Microplate reader
   Model: Optika, Mikura Ltd., UK
3. Block heater
   Rotilabo®-block thermostat. Model: H 250, Carlroth, Germany
4. Ultra-low Temperature Freezers
   Innova® Ultra-low Temperature Freezers, Model U101, USA
5. Centrifuge machine
   Eppendorf, Model-5430R, Hamburg, Germany)
6. Autoclave
   ALP Co. Ltd. KT-30L, Tokyo, japan
7. Refrigerator ($4^{\circ}C$)
   Bangladesh
8. Ice Machine
   Model AF-80, Scotsman, Italy
9. Aneroid sphygmomanometer
   ALPK2, Japan
10. Digital electric balance
    AUW-D Series, Shimadzu, Japan
11. Incubator
    Gallenkamp, UK
12. Ceramic blade cutter
    Japan
13. Forceps
    Pakistan

14. Polypropylene bottles
    Bangladesh
15. Eppendorf tubes
    Watson, Japan
16. Micropipettes
    Eppendorf, Germany
17. Micropipette tips
    Watson, Japan
18. Digestion vessel (Polypropylene)
    Santa Cruz, USA
19. Multichannel Pipettes
    Eppendorp, Germany
19. Beakers
    Pyrex, Germany
20. Volumetric flasks
    Pyrex, Germany
21. Measuring cylinders
    Pyrex, Germany
22. Test tubes
    Pyrex, Germany
23. Distilled water bottles
    Bangladesh
24. Conical flasks
    Pyrex, Germany
27. Weight machine
    China
28. Cryotubes
    TPP, Switzerland
29. Syringes
    Bangladesh

30. Blood Collection Tubes
    China
31. Vinyl gloves
    China
32. Aluminum foil
    Bangladesh
33. Scotch Tape
    Bangladesh
35. Measuring tape
    China
36 Sample storage box
    China

(A)

(B)

(C)

(D)

(E)

Figure 2.1 Major laboratory equipments used for this study. ICP-MS **(A),** Block heater **(B),** Microplate reader **(C),** Ultra-low Temperature Freezers **(D)** and Centrifuge machine **(E)**

Chapter 3

ASSOCIATION OF ARSENIC EXPOSURE WITH SERUM IMMUNOGLOBULIN E

CHAPTER 3: ASSOCIATION OF ARSENIC EXPOSURE WITH SERUM IMMUNOGLOBULIN E

3. Abstract

Immunoglobulin E (IgE) is a hallmark of bronchial asthma. Although arsenic exposure has been reported to be associated with asthma, effects of arsenic exposure on IgE have not yet been documented clearly. Therefore, this section of the study was designed to explore the associations of arsenic exposure with circulating IgE levels. A total of 260 study subjects, 194 from arsenic-endemic and 66 from non-endemic areas in Bangladesh were selected. Arsenic concentrations in drinking water, hair and nails of the study populations were measured by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) and serum immunoglobulin E (IgE) levels were measured by IgE ELISA kit through micro plate reader. In this study, we found that serum IgE levels (1372.55 ± 96.74 IU/ml) were significantly ($p < 0.001$) higher in arsenic endemic population than those (723.08 ± 73.57 IU/ml) in non-endemic population. Further serum IgE levels showed significant positive associations with arsenic concentrations in drinking water ($r_s = 0.220$, $p < 0.001$), hair ($r_s = 0.215$, $p < 0.001$) and nails ($r_s = 0.175$, $p < 0.01$) of the study individuals. In multiple regression analyses, only arsenic exposure metrics (drinking water, hair and hair nails) but not other variables (age, sex, BMI, smoking and socioeconomic conditions) showed significant associations with serum IgE levels. Finally, arsenic concentrations in drinking water, hair and nails showed novel dose-responses relationships with serum IgE levels. Thus, the increased levels of serum IgE observed in this study in arsenic-endemic population might be involved in the allergic reactions leading to bronchial asthma.

3.1 Introduction

In the arsenic-endemic areas of the world, the main cause of human exposure to arsenic is the drinking of water contaminated by arsenic, and arsenic toxicity through drinking water represents one of the biggest catastrophes in history, affecting millions of people in the world (BGS and DPHE, 2001; WHO, 2001). Bangladesh is one of the most severely affected regions in that approximately 80 million people consume water containing arsenic levels greater than the 10 μg/L standard set by the World Health Organization (Caldwell et al., 2003; Chowdhury, 2004). A significant number of toxicity cases have been already reported in the north-west region in Bangladesh and millions more are at risk for arsenic toxicity in the country (BGS and DPHE 2001; Chowdhury et al., 2000; Khan et al., 2003; Mukherjee and Bhattacharya, 2001). The situation is deteriorating as the new cases of arsenic toxicity are still being reported in different parts of the country. Arsenic toxicity induces dermatitis, multi-site cancers, cardiovascular diseases, diabetes mellitus, immune disorders, peripheral neuropathy, liver damage, renal failure, and other ailments (Chen et al., 2007; Huda et al., 2014; Karim et al., 2013; Mazumder et al., 1998; Mazumder, 2005; Meliker et al., 2007; Mumford et al., 2007; Tapio and Grosche 2006; Vahidnia et al., 2008; Wang et al., 2002). Ingestion of inorganic arsenic for a prolonged period also reported to cause respiratory problems including bronchial asthma (Islam et al., 2007; Mazumder et al., 2000; Saha, 1995, 2003; Saha et. al., 1999). Saha et al. (1995) conducted a study in West Bengal of India, and found a good number of patients with asthmatic symptoms in arsenic-endemic areas. They concluded that bronchitis and asthma were the common complications of ground water arsenic toxicity. Another study conducted in West Bengal of India also demonstrated a high rate of respiratory symptoms associated with asthma (Mazumder et al., 2000) in arsenic-endemic people. Islam et al. (2007) studied the link between arsenic exposure via drinking water and respiratory complications, and found higher prevalence of patients with asthmatic symptoms in arsenic-exposed population as compared to the un-exposed control population. All these epidemiological studies suggest that chronic human exposure to arsenic is associated with asthma.

Asthma is a common chronic inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction and

bronchospasm. Several genetic and non-genetic factors are responsible for the development of asthma (Sengler et al., 2002; Martinez, 1997). Most of the asthmatic cases are atopic in nature, with the trigger for acute asthma attacks. Elevated level of specific serum IgE towards common environmental allergen is a key component in the pathogenesis of atopic or allergic asthma**. (**D'Amato et al., 2014). IgE, the fifth immunoglobulin isotype consists of two heavy chains (ε chain) and two light chains. IgE has also been reported to be a risk factor of non-allergic asthma (Beeh et al., 2000). IgE causes chronic airway inflammation through effector cells such as mast cells, basophils etc, activated via high-affinity (Fcε RI) or low-affinity (Fcε RII) IgE receptors. Because of its central role in the pathogenesis of bronchial asthma, monoclonal antibody against IgE has been developed to treat or prevent asthma (D'Amato et al., 2014; Djukanović et al., 2004; Holgate and Polosa, 2008). Although several epidemiological studies have reported that chronic exposure to arsenic is associated with asthmatic symptoms, underlying mechanism is unknown. This study was designed to explore the association of arsenic exposure with serum IgE levels in human individuals exposed to arsenic chronically in Bangladesh.

3.2 Materials and Methods

3.2.1 Ethical permission

Ethical permission was taken from the Institute of Biological Sciences, University of Rajshahi, Bangladesh (41/320/IAMEBBC/IBSC). The subjects who participated in this study gave their written consent (annexure-I) and all sorts of confidentialities and rights of the study subjects were strictly maintained.

3.2.2 Study areas and study subjects

The arsenic-endemic and non-endemic areas and study subjects were selected as described previously (Ali et al., 2010; Hossain et al., 2012; Karim et al., 2010). Arsenic-endemic study 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 districts. Chowkoli, a village in Naogaon district with no history of arsenic contamination was selected as non-endemic area (Figure 3.1). Arsenic-endemic study areas were chosen according to the British Geological Survey report (2001). However, more detailed information on the location of the endemic-villages was taken from the local health offices. We found that, all families did not have their own drinking water (tube well water) sources. In many cases, a group of families used drinking water from one tube well. We recruited our study subjects based on the largest possible number of family members or families who used water from the selected tube wells. Many local residents had typical skin symptoms of arsenicosis (melanosis, hyperkeratosis, and hard patches on the palms of the hands and soles of the feet). Study subjects were selected irrespective of their skin symptoms. Those who responded spontaneously were asked to convene at a specific location in their village for initial screening purpose in light of exclusion criteria. The adults (15 to 60 years of ages) who had lived for at least last five years in the arsenic-endemic and non-endemic areas were recruited for this study. During the sample collection process, we were blinded to arsenic levels in drinking water, and to those in the hair and nails of the study participants. Attempts were 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 ratios of endemic and non-endemic subjects were approximately 3:1, and male and female

ratios in both endemic and non-endemic areas were also approximately 1:1. Endemic and non-endemic study subjects were individually matched on age (±5 years).

STUDY AREAS

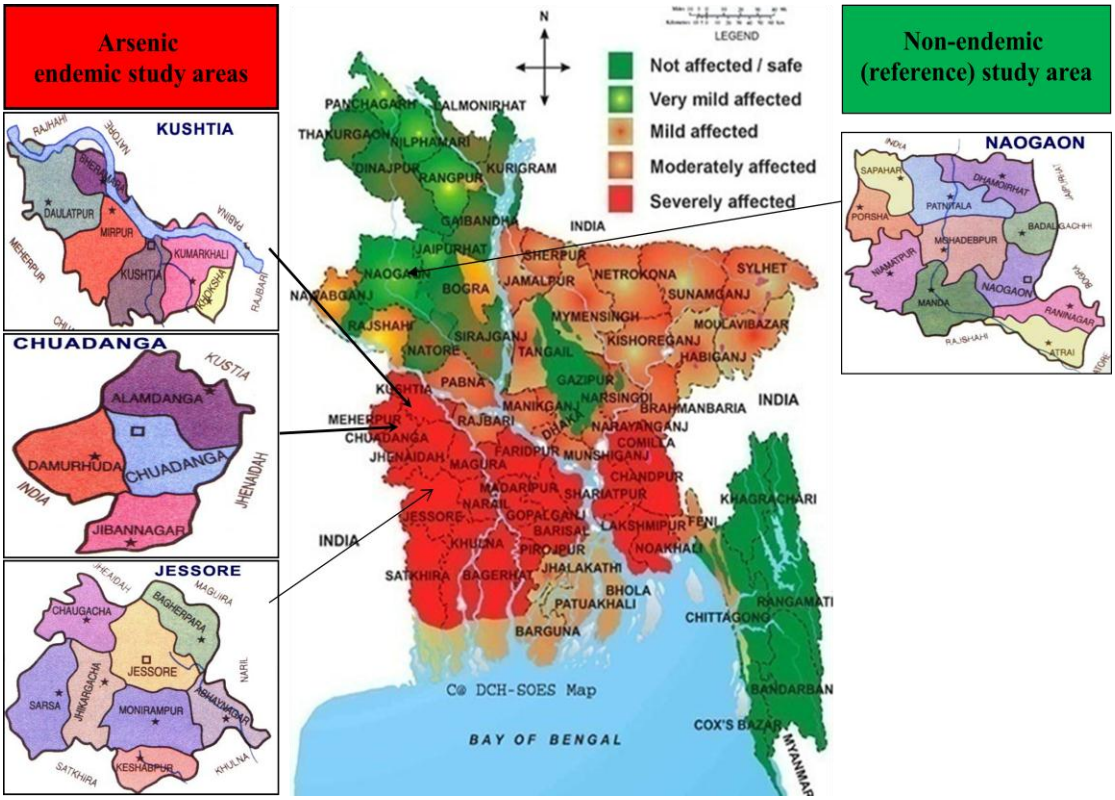

Figure 3.1 Arsenic-endemic (left panel) and non-endemic (right panel) areas in Bangladesh selected for this study

Source: [Groundwater Arsenic condition in GMB Plain at International Arsenic Conference (1998) by SOES-DCH at Dhaka, Bangladesh.
Available at: http://www.soesju.org/arsenic/alb_37.html]

Pregnant and lactating women, hepatitis B positive, and the individuals who had a history of drug addiction, chronic alcoholism, prescription of hepatotoxic and anti-hypertensive drugs, malaria, kalazar, and hepatic, renal or cardiac diseases were excluded from this study. Of the 203 individuals who were approached, 7 were excluded and 196 were primarily recruited in the arsenic-endemic areas. Finally, we further excluded 2 individuals after the collection of blood samples as they were found to be hepatitis B positive in our laboratory test. In non-endemic area, 4 from 70

individuals were excluded. We did not find any hepatitis B positive individuals in our laboratory test in non-endemic area.

The subjects participated in this study gave their written consent. 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 source of water for drinking and daily house hold 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. A copy of questionnaire has been included in annexure-II. Some photographs of field activities are shown bellow.

A **B**

C **D**

E **F**

Figure 3.2 Some representative photographs of the field activities (A) Personal interview of the study subjects, **(B)** Examination of a study subject by a physician **(C)** Measurement of BMI, **(D)** Collection of drinking water samples, **(E)** Nail and hair sample collection and **(F)** Blood collection.

3.2.3 Water collection and arsenic analysis

Water samples were collected from the tube wells which the study subjects are using as a primary source of drinking water as described previously (Ali et al., 2010; Islam et al., 2011). Water samples were collected on the same day when hair, nail, and blood samples of the study subjects were collected. After the tube well was pumped for five minutes (van Geen et al., 2008), the water samples from these tube wells were collected in acid-washed containers. Total arsenic concentration in water samples was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) after the addition of a solution of yttrium (10 µg/L in 1.0% nitric acid). Accuracy of ICP-MS determination of water arsenic concentration was confirmed using a CRM of 'River water' (NMIJ CRM 7202-a No.347 National Institute of Advanced Industrial Science and Technology, Japan). 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).

3.2.4 Collection of hair and nail samples, and analysis of arsenic

Hair and nail samples were collected from each study subject as described previously (Ali et al., 2010; Islam et al., 2011). Hair samples with the length of about 1 cm were collected from the region of the head close to scalp behind the ear. Nail were collected from the toes of each study subjects. Both hair and nail were washed by the method described by Chen et al. (1999). Samples were immersed in 1% Triton X-100, sonicated for 20 min, and then washed five times with milli-Q water. 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 μgL^{-1} as an internal standard for Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) (HP- 4500, Agilent Technologies, Kanagawa, Japan) analysis. The concentrations of total arsenic and yttrium in these samples were determined by ICP-MS. The ion signals of arsenic and yttrium were monitored at m/z of 75 and 89, respectively. All samples were determined in triplicate and the average values were used for data analysis. The detection limit of arsenic was 30 ng/L. Accuracy of arsenic measurement was verified using a certified reference material (CRM) of ''human hair'' (GBW09101, Shanghai Institute of Nuclear Research Academia Sinica, China).

The average value (mean ± SD) of arsenic in 'human hair' determined in triplicate by the above-mentioned digestion followed by ICP-MS analysis was 0.61 ± 0.12 μg/g (reference value, 0.59 μg/g).

3.2.5 Collection of blood serum

All study subjects were requested to fast overnight (10-12 h) to collect fasting blood samples. Blood samples (5-7 ml) were left at room temperature for 30 min for clotting and were subsequently centrifuged at 1,200 ×g for 20 min. The serum supernatant was then taken and stored at -80°C

3.2.6 Measurements of serum IgE

The IgE quantitative test is based on a solid phase enzyme-linked immunosorbent assay (ELISA). Serum IgE levels were measured by a commercially available sandwich enzyme immunoassay kit (DRG International, Inc., USA) according to the manufacture's protocol. On completion of the assay, the observed color change was measured using a microplate reader (Mikura Ltd. UK) and IgE levels were calculated by extrapolation from a standard curve. A standard curve was constructed for the immunoassay using the supplied IgE reference standards (0, 10, 50, 100, 400, 800 IU/mL igE in bovine serum). All standards and samples were analyzed in duplicate and the mean values were taken. In every assay the inter assay variations were maximum adjusted. According to the manufacturer's protocol, the minimum detectable concentration of IgE by this assay is estimated to be 5.0 IU/ml.

3.2.6.1 Principles of the IgE assay

The IgE quantitative test is a solid phase enzyme linked immunosorbent assay (ELISA) based on sandwich principle. The test specimen (serum) is added to the IgE monoclonal antibodies immobilized on polystyrene microtiter wells (solid phase) and incubated with the zero buffer. If human IgE is present in the specimen, it will combine with the antibodies on the well. The well is then washed to remove any residual test specimen, and goat anti-IgE in the antibody-enzyme (horseradish peroxidase) conjugate reagent is added. The conjugate reagent will bind immunologically to the IgE on the well, resulting in the IgE molecules being

sandwiched between the solid phase and enzyme-linked antibodies. After incubation at room temperature, the solid phase is washed with water to remove unbound-labeled antibodies. A solution of TMB reagent is added and incubated at room temperature for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of stop solution, and the color is changed to yellow and measured spectrophotometrically at 450 nm. The concentration of IgE is directly proportional to the color intensity of the test sample.

3.2.6.2 Constituents of human IgE ELISA kit

- Monoclonal anti-IgE coated microtiter plate
- Zero buffer
- Enzyme conjugate reagent
- IgE reference standards, containing 0, 10, 50, 100, 400, and 800 lU/ml (WHO, 2nd IRP 75/502).
- TMB reagent
- Stop solution

3.2.6.3 Stepwise assay

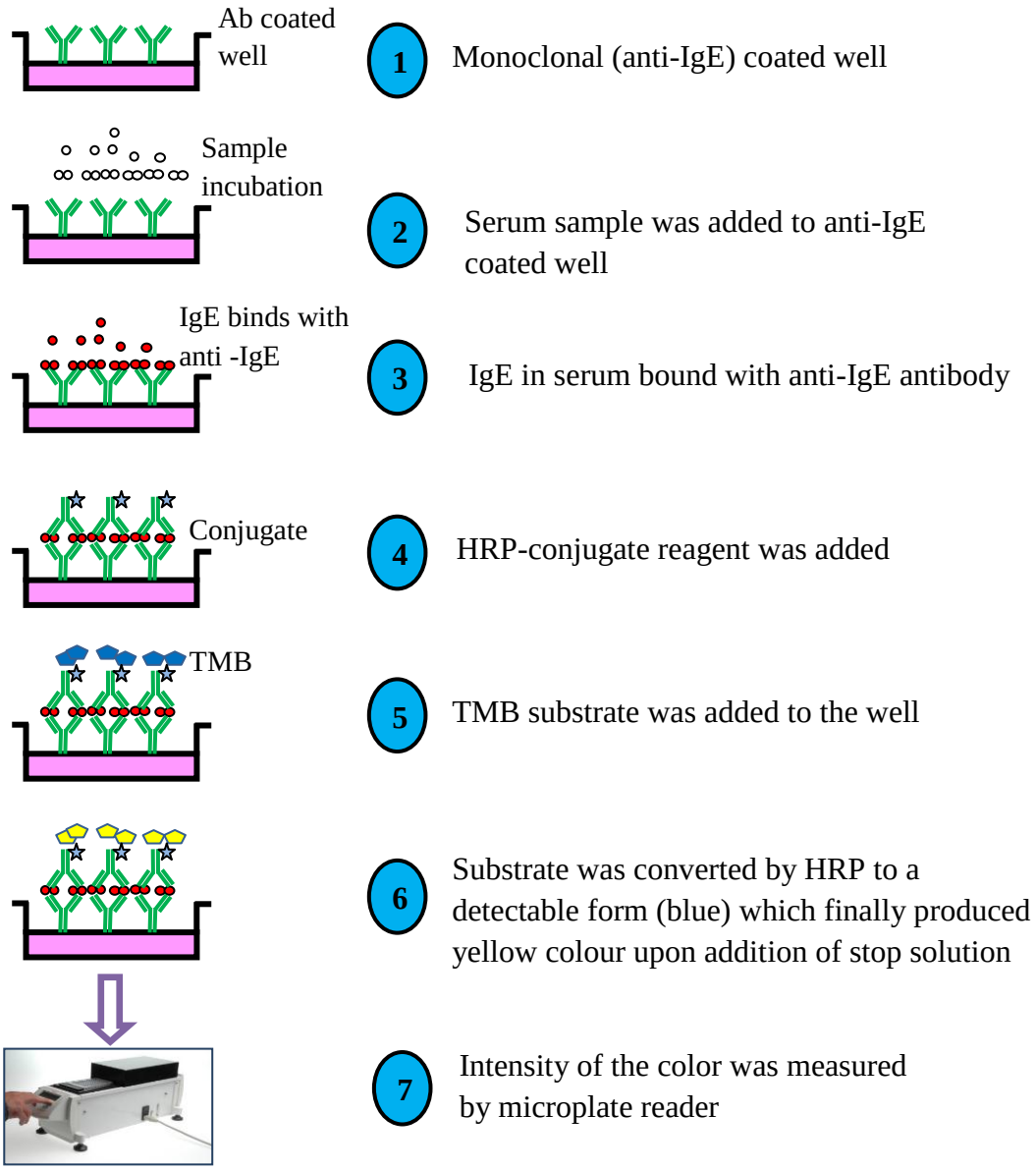

Figure 3.3 Stepwise assay of serum IgE by IgE Elisa kit

3.2.7 Statistical analysis

Statistical analyses for the study were performed using the Statistical Packages for Social Sciences (SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp) software. Due to the skewed distribution of the raw data, log transformed values of water, hair and nail arsenic, and levels of serum IgE were used. Normality of the distributions of variable was verified by Q-Q plot. The mean age, arsenic concentrations, monthly income and BMI of the study subjects in arsenic-endemic and non-endemic areas were compared by Independent Sample *T*-test, whereas the smoking status, occupation and educational status were compared by Chi-square test. Spearman correlation coefficient tests were used to evaluate the correlations of arsenic exposure metrics (drinking water, hair and nail arsenic concentrations) with serum IgE levels. Multiple regression analyses were performed to assess the effects of age, sex, BMI, smoking, occupation, income and education along with arsenic exposure metrics on serum IgE levels. The study subjects in arsenic-endemic areas were stratified through frequency test into 'low', 'medium' and 'high' exposure groups based on the three concentrations of arsenic in the drinking water, hair, and nails. The study subjects in the non-endemic area were used as a reference group ('lowest' exposure group). In addition, the study subjects were divided into three groups ($\leq$10 µg/L, 10.1-50 µg/L and > 50 µg/L) based on the regulatory upper limit of water arsenic concentration set by WHO (10 µg L^{-1}), and Bangladesh Government (50 µg L^{-1}). Finally, dose-response relationships of arsenic exposure metrics with serum IgE levels were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 General characteristics of the study subjects

Table 3.1 shows the characteristics of the study subjects in the arsenic-endemic and non-endemic areas. Of the total 260 participants, 194 were from arsenic-endemic areas and 66 from non-endemic area. Non-endemic population were selected for this study as a reference (control) group. Arsenic concentrations in drinking water, hair and nails of the study subjects in the arsenic -endemic areas were approximately 58, 15 and 6 times higher, respectively than in non-endemic control area. The average age of the study subjects in the arsenic-endemic and non-endemic areas were 38.90±13.41 and 37.12±12.45 years, respectively. In the arsenic-endemic areas, there were 99 male and 95 female study subjects, whereas in the non-endemic area, these were 34 and 32, respectively. Most of the male study subjects in both arsenic-endemic and non-endemic areas were farmers, whereas most of the female study subjects were housewives. Socioeconomic characteristics (occupation, education and monthly income) of the study subjects from non-endemic and endemic areas were almost similar. The percentage of tobacco smokers in the arsenic-endemic and non-endemic areas were 26.3 and 27.3, respectively. We did not find any female who admitted to be a smoker in the arsenic-endemic and non-endemic areas as generally Bangladeshi women do not smoke cigarette. The mean BMI of the study subjects in the arsenic endemic and non-endemic areas were 20.91±3.69 and 21.47±2.72, respectively.

Table 3.1 Descriptive characteristics of the study subjects in arsenic-endemic and non-endemic areas

Parameters	All subjects	Non-endemic	As-endemic	*p*-value[Ω]
Total subjects (n)	260	66	194	
Sex (n)				0.946[†]
Male	133	34	99	
Female	127	32	95	
Age (mean ± SD)	38.45±13.17	37.12±12.45	38.90±13.41	0.327*
As conc. in water (mean ± SD; µg/L)	112.41±140.43	2.57±2.38	149.78±144.68	< 0.001*
As conc. in hair (mean ± SD; µg/g)	3.66±5.01	0.32±0.21	4.79±5.34	< 0.001*
As conc. in nails (mean ± SD; µg/g)	7.41±7.16	1.49±1.16	9.43±7.23	< 0.001*
Occupation [n, (%)]				
Male				
Farmer	112(84.2)	28(82.4)	84(84.8)	
Business	4(3.0)	1(2.9)	3(3)	
Student	8(6.0)	4(11.8)	4(4)	
Others[‡]	9(6.8)	1(2.9)	8(8.1)	
Female				0.463[†]
Housewives	123(96.9)	31(96.9)	92(96.8)	
Students	3(2.4)	1(3.1)	2(2.1)	
Others[‡]	1(.8)	0(0)	1(1.1)	
Education [n, (%)]				
No formal education	125(48.1)	36(54.5)	89(45.9)	
Primary	95(36.5)	24(36.4)	71(36.6)	0.201[†]
Secondary	30(11.5)	3(4.5)	27(13.9)	
Higher	10(3.8)	3(4.5)	7(3.5)	
Income/month [US$, (mean ± SD)]	24.11±7.82	23.70±6.30	24.25±8.29	0.579*
Smoking [n, (%)]				
Yes	69(26.5)	18(27.3)	51(26.3)	0.876[†]
No	191(73.5)	48(72.7)	143(73.7)	
BMI (mean ± SD; Kg/m^2)	21.05±3.47	21.47±2.72	20.91±3.69	0.261*

Abbreviations: As, Arsenic. Data were presented as mean ± SD. BMI was calculated as body weight (Kg) divided by height squared (m^2). **p*- and [†]*p*- values between arsenic-endemic and non-endemic areas were from the Independent Sample T-test and Pearson chi-square test, respectively. [‡]Others included tailors, rickshaw puller, security guard and retired worker.

3.3.2 Interrelation between drinking water, hair and nail arsenic concentrations.

First we checked the correlation of external arsenic exposure metric (drinking water) with the internal arsenic exposure metrics (hair and nail) of the study individuals. Drinking water arsenic concentrations showed a strong positive relationship (r_s = 0.713, p < 0.001) with hair arsenic concentrations (Figure 3.4A). Almost similar shape of relationship (r_s = 0.722, p < 0.001) was also observed between water and nail arsenic concentrations (Figure 3.4B). Finally, we checked the correlation of hair arsenic concentrations with nail arsenic concentrations, and as it was expected, hair arsenic concentration also showed a strong positive relationship (r_s = 0.823, p < 0.001) with nail arsenic concentrations (Figure 3.4C).

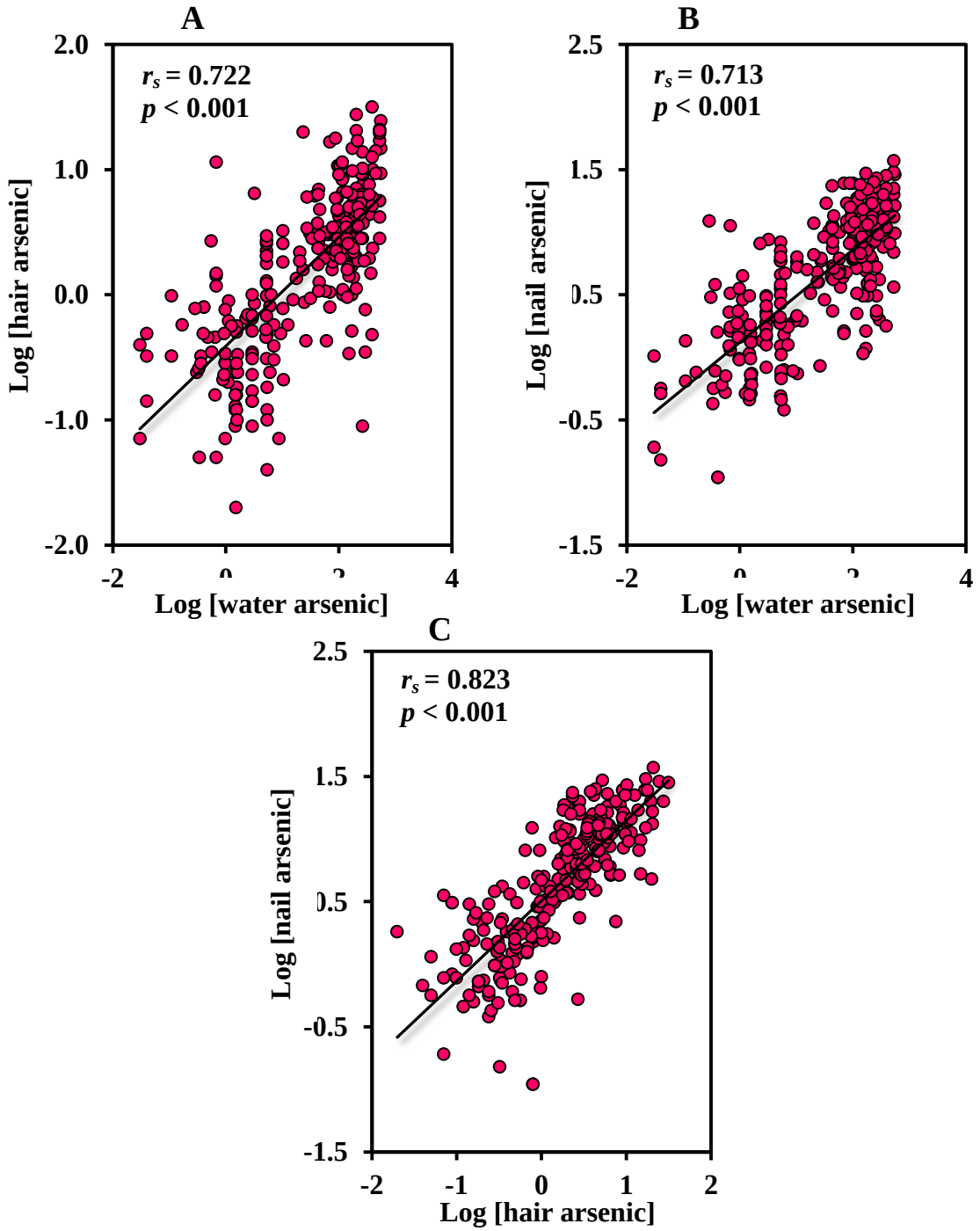

Figure 3.4 Interrelation between drinking water, hair and nail arsenic concentrations. (A) Correlation between drinking water and hair arsenic concentrations, **(B)** Correlation between drinking water and nail arsenic concentration of the study subjects. **(C)** Correlation between hair and nails arsenic concentration of the study subjects. Log transformed value of arsenic concentrations in water, hair and nails were used. Interrelationships between drinking water and hair, between drinking water and nails, and between hair and nail arsenic concentrations were significantly positive. r_s and p-values were from Spearman correlation coefficient test.

3.3.3 Comparison of serum IgE levels between arsenic-endemic and non-endemic populations

Figure 3.5 shows the comparison of serum IgE levels in arsenic-endemic and non-endemic populations. It was observed that serum IgE levels were significantly higher ($p < 0.001$) in arsenic-endemic (1372.55 ± 96.74 IU/ml) study population than in non-endemic (723.08 ± 73.57 IU/ml) study population.

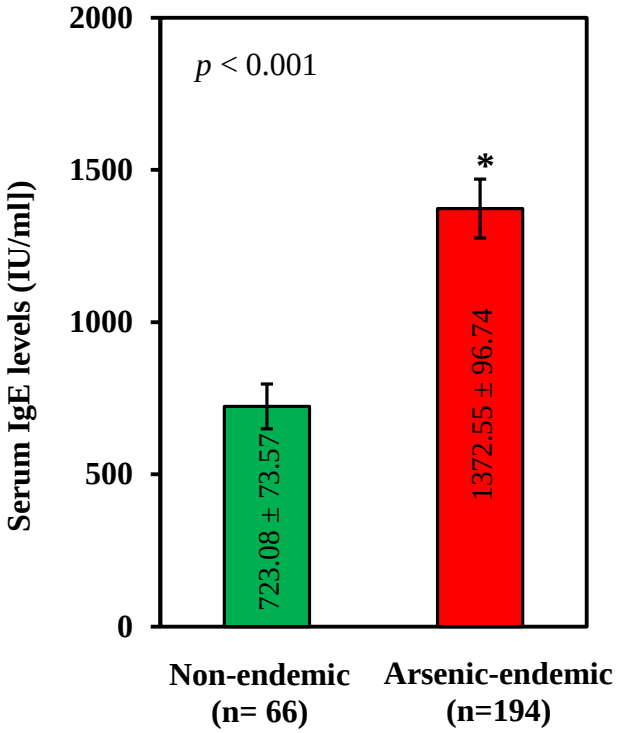

Figure 3.5 Comparison of serum IgE levels between arsenic-endemic and non-endemic populations. Green and red columns represent serum IgE levels (mean ± SE) of the study subjects in non-endemic and arsenic-endemic areas, respectively. IgE levels in non-endemic and endemic populations were 723.08 ± 73.57 IU/ml and 1372.55 ± 96.74 IU/ml, respectively. *Significantly different at $p < 0.001$. *p*-values were from Independent Sample *T*-test.

3.3.4 Correlation of serum IgE levels with arsenic exposure metrics

Figure 3.6 shows the correlations of serum IgE levels with drinking water, hair and nail arsenic concentrations of the study subjects. Serum IgE levels showed a significant (r_s = 0.220, p < 0.001) positive correlation with arsenic concentrations in drinking water (Figure 3.6A). Serum IgE levels also showed significant positive associations with arsenic concentrations in hair (r_s = 0.215, p < 0.001) and nails (r_s = 0.175, p < 0.01) (Figure 3.6B, C).

3.3.5 Multiple regression analyses for the factors associated with serum IgE levels

Table 3.2 shows the multiple regression analyses for the factors or variables associated with serum IgE levels. In this study, arsenic exposure, age, sex, BMI, smoking and socioeconomic conditions (occupations, income and educational status) were considered as variables or factors that might influence the serum IgE levels. Regression analyses showed that only arsenic exposure (drinking water, hair and nail arsenic concentrations) but not other variables were associated the elevation of serum IgE levels.

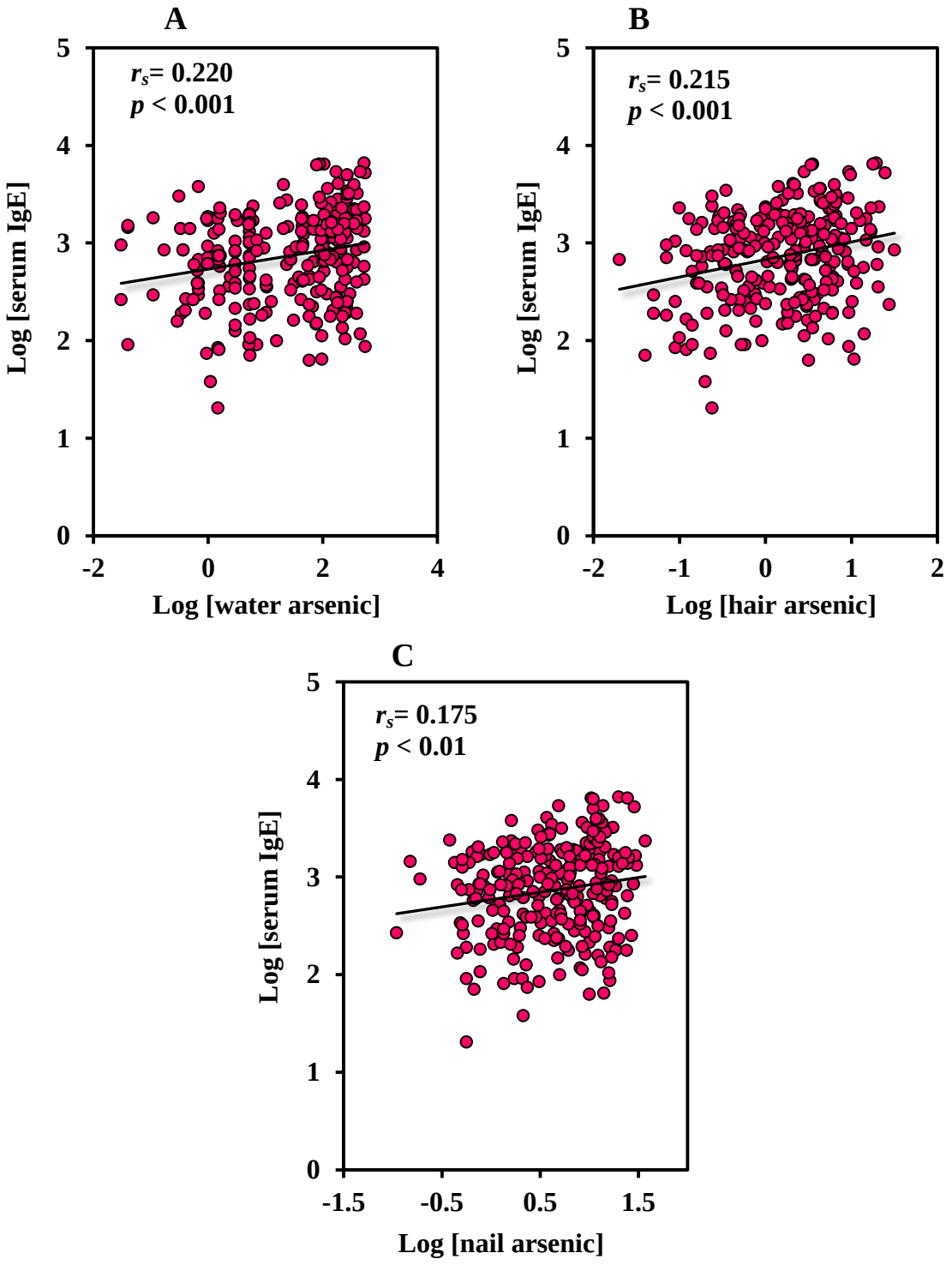

Figure 3.6 Correlations of serum IgE levels with arsenic exposure metrics Correlations of drinking water **(A)**, hair **(B)** and nail **(C)** arsenic concentrations with serum IgE levels. Serum IgE levels were significantly and positively associated with the arsenic concentrations in drinking water, hair and nails of the study individuals. Log transformed values of arsenic concentrations and serum IgE levels were used. r_s and *p*-values were from Spearman correlation coefficient test.

Table 3.2 Multiple regression analyses for the factors associated with serum IgE levels

	Dependent variable (Serum IgE)	
Independent variables	**β-Coefficient**	***p*-value**
Water arsenic	0.097	< 0.001
Age	0.002	0.417
Sex	-0.051	0.509
BMI	-0.004	0.684
Smoking	-0.058	0.475
Occupation	-0.051	0.149
Income	-0.008	0.115
Education	0.039	0.317
Hair arsenic	0.183	< 0.001
Age	0.002	0.487
Sex	-0.057	0.461
BMI	-0.001	0.915
Smoking	-0.072	0.371
Occupation	-0.048	0.173
Income	-0.008	0.115
Education	0.032	0.409
Nail arsenic	0.157	< 0.01
Age	0.002	0.443
Sex	-0.055	0.480
BMI	-0.002	0.814
Smoking	-0.060	0.461
Occupation	-0.053	0.138
Income	-0.008	0.129
Education	0.032	0.410

Log-transformed values of arsenic concentrations in exposure metrics and serum IgE levels were used.

3.3.6 Dose-response relationships of arsenic exposure with serum IgE levels

To investigate the dose-response relationship between arsenic exposure and serum IgE levels, the study subjects were split into four groups (lowest, low, medium and high) based on the concentrations of arsenic in drinking water, hair and nails, where study subjects of the non-endemic area were used as a lowest exposure (reference) group. Intriguingly, serum IgE levels were found to be higher in higher exposure gradients of water, hair and nail arsenic concentrations. Although gradual increases of serum IgE levels were observed in the higher concentration gradients compared to the lower gradients of water arsenic, the differences were significant in the high versus lowest ($p < 0.001$) and high versus low ($p < 0.01$) arsenic exposure groups (Figure 3.7 A). Almost similar shape of dose-response relationships of serum IgE levels with hair and nail arsenic gradients were observed as it was observed in the water arsenic gradients. In the case of hair arsenic gradients, serum IgE levels were also significantly higher in the high exposure groups ($p < 0.01$) as compared to the lowest and low exposure groups (Figure 3.7 B), whereas in the case of nail arsenic gradients, the differences were significant in the high versus lowest ($p < 0.01$) and the high versus low ($p < 0.05$) arsenic exposure groups (Figure 3.7 C).

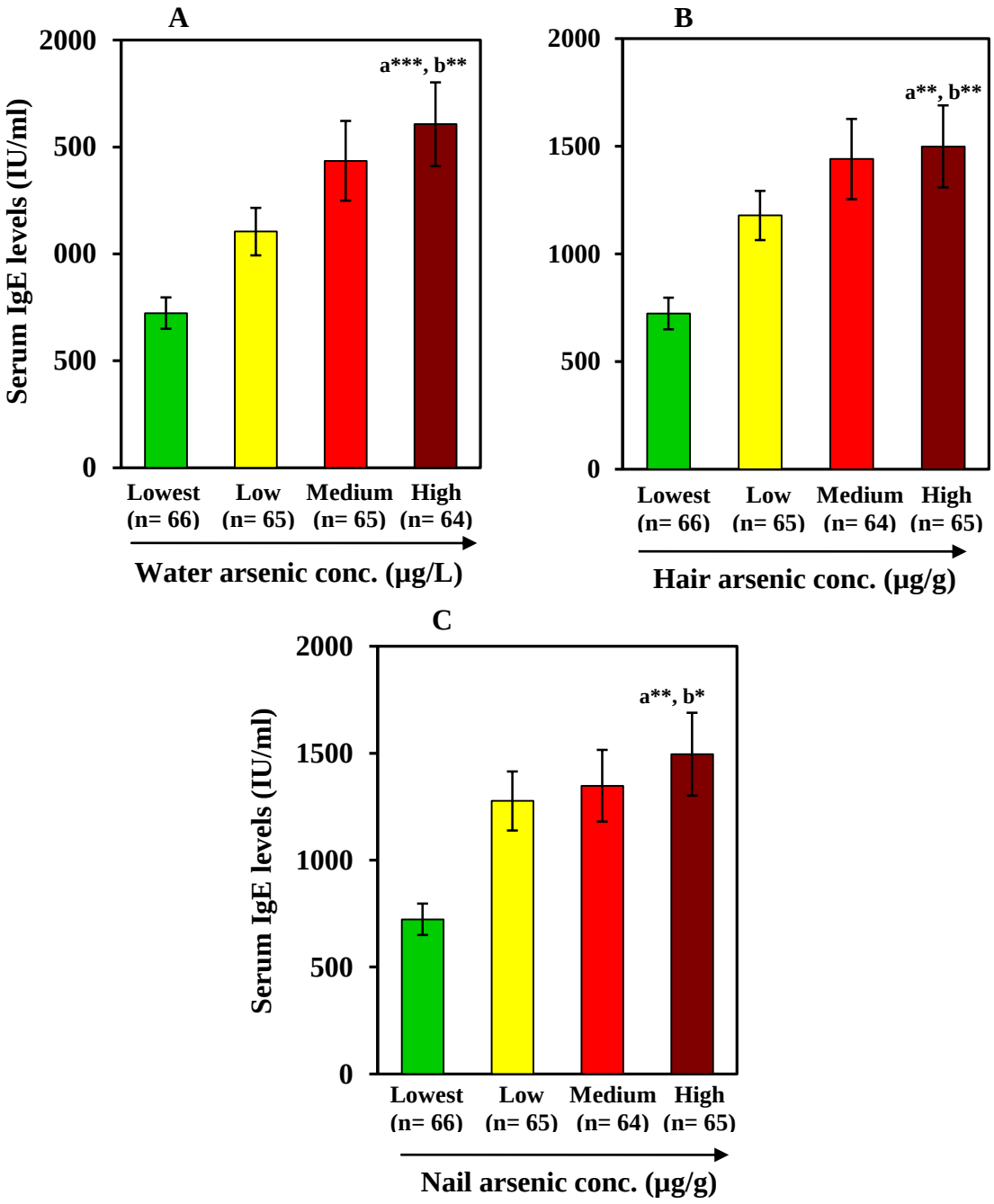

Figure 3.7 Dose-response relationships of arsenic exposure with serum IgE levels. Green, yellow, red and dark red columns represent the serum IgE levels (mean ± SE) of lowest, low, medium and high exposure groups of study subjects, respectively. The groups based on drinking water arsenic levels were lowest (0.03-10.12 µg/L; n=66), low (0.11- 45.80 µg/L; n=65), medium (45.81-180.00 µg/L; n=65), and high (180.01 – 546 µg/L; n=64). The groups based on hair arsenic levels were lowest (0.02 – 1.00 µg/g; n=66), low (0.05 – 1.96 µg/g; n=65), medium (1.97–4.65 µg/g; n=64), and high (4.66 –31.81 µg/g; n=65). The groups based on nail arsenic levels were lowest (0.15–8.13 µg/g; n=66), low (0.11 – 4.92 µg/g; n=65), medium (4.93 – 11.0 µg/g; n=64), and high (11.01 – 37.42 µg/g; n=65). In the all cases, lowest groups were recognized as the reference group. All *p*-values were from one-way ANOVA. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. [a] Significantly different from lowest group. [b] Significantly different from low group.

3.3.7 Serum IgE levels in the three groups based on the regulatory upper limit of drinking water arsenic concentrations

The study subjects were split 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 serum IgE levels (Figure 3.8). The serum IgE levels (mean ± SE) in the ≤10 µg/L, 10.1-50 µg/L and > 50 µg/L groups of study subjects were 845.70 ± 73.12 IU/ml, 1077.97 ± 161.62 IU/ml and 1511.52 ± 131.94 IU/ml, respectively. Serum IgE levels in > 50 µg/L group were found to be significantly ($p < 0.001$) higher than in ≤10 µg/L group. Although serum IgE levels were found to be higher in 10.1-50 µg/L group as compared to ≤ 10 µg/L group, the difference was not significant statistically.

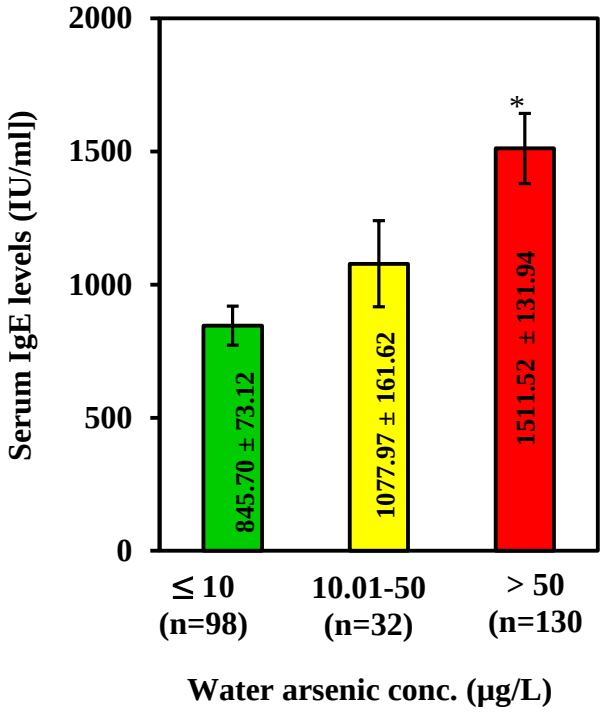

Figure 3.8 Serum IgE levels in the three groups based on the regulatory upper limit of drinking water arsenic concentrations. Green, yellow and red bars represent serum IgE levels (mean ± SE) of ≤ 10 µg/L (n=98), 10.1-50 µg/L (n=32) and > 50 µg/L (n=130) exposure groups of study subjects. Serum IgE levels in ≤10 µg/L, 10.1-50 µg/L and > 50 µg/L groups were 845.70 ± 73.12 IU/ml, 1077.97 ± 161.62 IU/ml and 1511.52 ± 131.94 IU/ml, respectively. *Statistically significant from ≤10 µg/L group at $p < 0.001$.

3.4 Discussion

There are two major forms of asthma: allergic (extrinsic) and non-allergic (intrinsic). Allergic (atopic) asthma is the most common form of asthma in both adult and children. Allergic asthma is mediated through a complex process involving several molecules and cells. Among molecules, IgE is a key component for allergic reactions leading to bronchial asthma. Although arsenic exposure has been reported to be associated with asthma, however, very little information are available regarding the association of arsenic exposure with circulating IgE. This epidemiological study showed the association of serum IgE levels with arsenic exposure recruiting the human subjects from both arsenic-endemic and non-endemic areas in Bangladesh.

Several epidemiological studies have consistently reported that patients with asthma have elevated levels of IgE compared to the non-asthmatic population (Burrows et al., 1989; Holford-Strevens et al., 1984; Grundbacher and Massie, 1985; Wittig et al., 1980; Sears et al., 1980). Increased IgE level is a hallmark of allergic-mediated asthma. The mechanism of allergic asthma is largely mediated though the activation of mast cells and basophil cells. Mast cells and basophil cells are activated by cross-linking of IgE receptor (FcεRI) which occurs by binding of multivalent antigens to the attached IgE molecules. Receptor cross linking by IgE causes the activation of several intracellular signals for triggering three types of biologic responses: secretion of the preformed contents of their granules mainly histamine, synthesis and secretion of lipid mediators, and synthesis and secretion of cytokines that ultimately lead to the initiation of allergic inflammation and bronchial spasm (Abbas and Litchman, 2005).

There are contradictory findings about the effect of arsenic on IgE production. Chu et al. (2010) reported that arsenic treatment did not change the serum IgE levels in antigen-sensitized mice. In contrast, Islam et al. (2007) showed that serum IgE levels were higher in arsenic-exposed people than the control (non-exposed) people. These discrepancies might be due to the differences in experimental model used. Results of animal model experiments often cannot be translated to human because of the complications in conversion of exact dose, duration of exposure, and species specific metabolic and genetic differences. In fact no suitable animal model for

arsenic toxicity has been developed yet that can well correspond to the toxic effects of arsenic exposure observed in humans. Discrepancies in the results in animal and human studies necessitate more systematic human studies that can provide detail information about the effects of chronic arsenic exposure on serum IgE levels. Therefore, findings of our study are noteworthy. Significantly higher levels of IgE in arsenic-exposed people compared to the un-exposed people found in the study conducted by Islam et al. (2007) were in good agreement with results of our study in which we also found that serum IgE levels were significantly higher in arsenic-endemic population than in non-endemic population (Figure 3.5). Additionally, to the best of our knowledge, this study for the first time showed the dose-response relationship between arsenic exposure and serum IgE levels (Figure 3.7 and Figure 3.8). This result is particularly important since dose-response relationship indicates the safe and toxic doses of any substance.

Although many studies suggest that several variables are concerned with the elevated levels of IgE, there has not been general agreement about the effects of demographic or other variables on serum IgE levels. Major factors that can influence the allergic asthma as well as serum IgE levels are age, sex, BMI, smoking and socioeconomic condition (Barbee et al., 1987; Bergmann et al., 2000; Criqui et al., 1990; Huang et al., 1999; Litonjua et al., 2005; Omenaas et al., 1994; Visness et al., 2009). In this study, we considered those variables as possible confounders along with arsenic exposure that could change the serum IgE levels. Intriguingly, in multiple regression analyses, we found that only drinking water, hair and nail arsenic concentrations but not other variables showed significant effects on the alteration of serum IgE levels (Table 3.2). These results argued that arsenic exposure was a main contributor for the elevation of serum IgE levels of the study individuals.

Asthma is a major problem that decreases the quality of life interfering the daily activities. Treatment of chronic asthma is costly and it makes a big economic burden of the patients. Even severe asthma attack can make a life-threatening condition. The increases in asthma symptom prevalence in Africa, Latin America and Asia indicate that the global burden of asthma is continuing to rise (Pearce et al., 2007). Therefore, even a little effect of chronic exposure to arsenic on the development of asthmatic symptoms may further aggravate the situation. There are several unique features in

the present study. First, all the associations of serum IgE levels were found across the three kinds of exposure metrics (water, hair and nail arsenic concentrations) (Figure 3.6). In our previous study, we found that drinking water arsenic concentrations were strongly correlated with hair and nail arsenic concentrations (Ali et al., 2010; Karim et al., 2013). We performed this study on the same population groups and almost similar correlations were observed among the exposure metrics (Figure 3.4). Therefore, the assessment of arsenic exposure by three kinds of exposure metrics and their positive correlations with serum IgE levels might exclude the possibilities of miss classification of the study subjects. Second, this study had a good number of study subjects with a wide variation of arsenic exposure levels that showed a precise nature of dose-response relationship between arsenic exposure and serum IgE levels (Figure 3.7). Further dose-response relationship among the three groups (≤10 µg/L, 10.1-50 µg/L and >50 µg/L) based on the permissive limit of water arsenic set by WHO and Bangladesh Government showed the higher levels of serum IgE levels in 10.1-50 µg/L than in ≤10 µg/L groups (Figure 3.8). Although the difference was not statistically significant, the result might be of important note from policy perspectives as the permissive limit set by the Bangladesh government is 5 times higher than that of the WHO.

There are several limitations to this study that should be noted. First, although we did not observe any significant effect of potential variables such as age, sex, BMI, smoking, socioeconomic conditions on serum IgE levels (Table 3.2), there might be some other factors such as co-exposure to other metals, insecticides and pesticides and individual nutritional status that could influence the serum IgE levels. If any accompanying metals or other cofactors would have effects on the observed associations, then they would also be expected to follow the same concentration gradients as the arsenic in the drinking water, hair and nails. This is unlikely, but more extensive study of the other metals and their association with serum IgE levels is required in future. Second, this study was designed to be cross sectional, but not prospective. A cohort based study is needed in future in order to verify the cause-effect relationship between arsenic exposure and serum IgE levels. Third, most of our study individuals were at the lower end of normal range of BMI (Table 3.1) and the socioeconomic status of the majority of the study subjects were poor. This may limit

the generalization of this study with other population. Therefore, findings of this study may not be reproducible to the other population if they do not adhere with characteristics of the population in this study.

3.5 Conclusions

This cross-sectional study demonstrated that serum IgE levels were significantly higher in arsenic-endemic population than in non-endemic population. Arsenic exposure (drinking water, hair and nail arsenic concentrations) of the study individuals showed significant positive associations with serum IgE levels. Finally, arsenic exposure showed a novel dose-response relationship with serum IgE levels. Thus the elevated levels of serum IgE observed in this study might be involved in the development of asthma in arsenic-endemic population.

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Chapter 4

ASSOCIATION OF ARSENIC EXPOSURE WITH SERUM INTERLEUKIN-4

CHAPTER 4: ASSOCIATION OF ARSENIC EXPOSURE WITH SERUM INTERLEUKIN-4

4. Abstract

Chronic exposure to arsenic has been reported to be associated with respiratory complications including asthma. Interleukin-4 (IL-4) is a key regulator for immunoglobulin E (IgE)-mediated allergic reactions leading to bronchial asthma. IL-4 has also been reported to be involved in the expression of several other molecules including vascular cell adhesion molecule-1 (VCAM-1) that are particularly implicated in allergic inflammation. However, the association between arsenic exposure and circulating IL-4 has not yet been documented. As shown in chapter 3, we found that arsenic exposure (drinking water, hair and nails arsenic) of the study populations had positive correlations with serum IgE levels. These results led us to explore the association of arsenic exposure with serum IL-4 levels on the same population groups for understanding the mechanism of arsenic-induced asthma. Serum IL-4 levels were measured by human IL-4 ELISA kit through micro plate reader. In this study, we found that serum IL-4 levels were significantly ($p < 0.05$) higher in arsenic-endemic population than in non-endemic population. Serum IL-4 levels were also found to be significantly associated with the arsenic concentrations in drinking water ($r_s = 0.184$, $p < 0.01$), hair ($r_s = 0.204$, $p < 0.01$) and nails ($r_s = 0.170$, $p < 0.01$) of the study individuals. Multiple regression analyses showed that only arsenic but not other relevant variables (age, sex, BMI, smoking and socioeconomic conditions) had significant effects on serum IL-4 levels. Furthermore, arsenic exposure metrics (drinking water, hair and nail arsenic) showed dose-response relationships with serum IL-4 levels. Intriguingly, serum IL-4 levels were positively associated with circulating IgE and VCAM-1 levels. Taken together, the results of this study indicate that arsenic exposure-related bronchial asthma may be mediated through IL-4-IgE-sensitive pathways.

4.1 Introduction

Chronic exposure to arsenic via drinking water, foods and occupation is a major threat to the public health worldwide, affecting hundreds of millions of people. Bangladesh has been grappling with the largest mass poisoning of a population in history because of the drinking of water contaminated by inorganic arsenic greater than the permissive limit (10µg/L) for the arsenic-endemic people set by World Health Organization (WHO) (Caldwell et al., 2003; Chowdhury, 2004). 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). Ingestion of inorganic arsenic has been documented to be associated with a variety of neoplastic and non-neoplastic diseases (Chen et al., 2007; Huda et al., 2014; Karim et al., 2013; Mazumder 2005; Mazumder et al., 1998; Meliker et al., 2007; Mumford et al., 2007; Tapio and Grosche 2006; Vahidnia et al., 2008; Wang et al., 2002). Several studies have reported that chronic exposure to arsenic causes respiratory complications including asthmatic symptoms (Islam et al., 2007; Mazumder et al., 2000; Saha, 1995, 2003; Saha et. al., 1999). Bronchial asthma is one of the most commonly observed inflammatory diseases worldwide including both developing and developed countries. 60% of asthma cases are "allergic-asthma" (NCEH, CDC, 1999). In recent decades number of asthmatic patients is being increased gradually across the all age, sex and racial groups. Asthma decreases the quality of life of the patients and also causes economical burden of the patients because of the costly and prolonged treatment procedure. In the worst cases, asthma may cause life-threatening condition. In the USA it has been estimated that each year 3300 Americans die from asthma. In addition, asthma is indicated as "contributing factor" for nearly 7,000 other deaths each year (NCEH, CDC, 2001). It is a complex disease involving several molecules, cells and various genetic and non-genetic factors. The major molecules involved in the allergic asthma are IgE and IL-4. IL-4 is a key cytokine in the development of allergic inflammation. IL-4 is a multifunctional immunoregulatory cytokine. It is a major stimulus for the production of IgE antibodies and for the development of T_H2 cells from naive $CD4^+$ helper T cells. IL-4 is the signature cytokine of the T_H2 subset and functions as both the inducer and an effector cytokine of these cells. In addition to

the T_H2 subset of $CD4^+$ T lymphocytes, IL-4 can also be produced from activated mast cells and basophils in response to receptor-mediated events (Abbas et al., 1996; Seder, 1994). IL-4 is important in initial sensitization to allergens and causes isotype switching (class switching) of B cells to IgE produing cells (Barnes, 2008; Holt et al., 1999). The produced IgE are then bind with Fcε receptors of mast cells and basophiles through their Fc regions and cause receptor cross-linking because of the binding of IgE molecules with the multivalent antigen. Cross-linking of receptors on mast cells and basophiles activates several signaling events leading to the degranulation and release of histamine, production and secretion of several lipid mediators and cytokines. These events subsequently cause chronic bronchial asthma-related lung remodeling (Doucet et al., 1998; Marone et al, 1997; Stone et al., 2010). An additional mechanism by which IL-4 contributes to airway obstruction in asthma through the upregulation of mucin gene expression resulting hyper secretion of mucus, common symptoms of bronchial asthma (Dabbagh et al., 1999). IL-4 has been reported to be involved in the inflammatory process of allergic asthma through the expression of VCAM-1 (Thornhill et al., 1991). Significant positive association of arsenic exposure with serum IgE levels observed in the previous chapter (Chapter 3) suggested the possible implication of IgE in arsenic exposure-related asthma. Since IL-4 is an important regulator for IgE and other mediators of allergic asthma, this section of the study has been designed to explore the association of arsenic exposure with serum IL-4.

4.2 Materials and Methods

4.2.1 Ethical permission

Ethical permission was taken as described in the materials and methods section of the previous chapter (Chapter 3).

4.2.2 Study areas and study subjects

Study areas and study subjects were chosen as described previously in the materials and methods section of the previous chapter (Chapter 3).

4.2.3 Collection of drinking water, hair and nail samples, and analysis of arsenic

Collection of drinking water, hair and nail samples from each study subject and analysis of the total arsenic concentrations in the samples were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as described in the materials and method section of the previous chapter (Chapter 3).

4.2.4 Collection of blood serum

Serum from blood samples were collected as described in the materials and methods section of the previous chapter (Chapter 3)

4.2.5 Measurements of serum IL-4

The IL-4 quantitative test is based on a solid phase enzyme-linked immunosorbent assay (ELISA). Serum IL-4 levels were measured by a commercially available sandwich enzyme immunoassay kit (R&D Systems, Inc., USA) according to the manufacture's protocol. On completion of the assay, the observed color change was measured using a microplate reader (Mikura Ltd. UK) and IL-4 levels were calculated by extrapolation from a standard curve. A standard curve was constructed for the immunoassay using the supplied IL-4 reference. All standards and samples were analyzed in duplicate and the mean values were taken. In every assay the inter assay variations were adjusted. According to the manufacturer's protocol, the minimum detectable dose (MDD) of IL-4 was less than 10 pg/mL.

4.2.5.1 Principles of the IL-4 assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human IL-4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells, and any IL-4 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human IL-4 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-4 bound in the initial step. The color development is stopped and the intensity of the color is measured.

4.2.5.2 Constituents of human IL-4 ELISA kit

The constituents of human IL-4 ELISA kit are listed in the Table 4.1

Table 4.1 Constituents of human IL-4 ELISA kit

Constituents	Description
IL-4 Microplate	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human IL-4.
IL-4 Standard	Recombinant human IL-4 in a buffered protein base with preservatives; lyophilized.
IL-4 Conjugate	Polyclonal antibody specific for human IL-4 conjugated to horseradish peroxidase with preservatives.
Assay Diluent	Buffered protein base with preservatives.
Calibrator Diluents	Animal serum with preservatives.
Wash Buffer	25-fold concentrated solution of buffered surfactant with preservative.
Color Reagent A	Stabilized hydrogen peroxide.
Color Reagent B	Stabilized chromogen (TMB).
Stop Solution	2N sulfuric acid.
Plate Sealers	Adhesive strips.

4.2.5.3 Stepwise assay

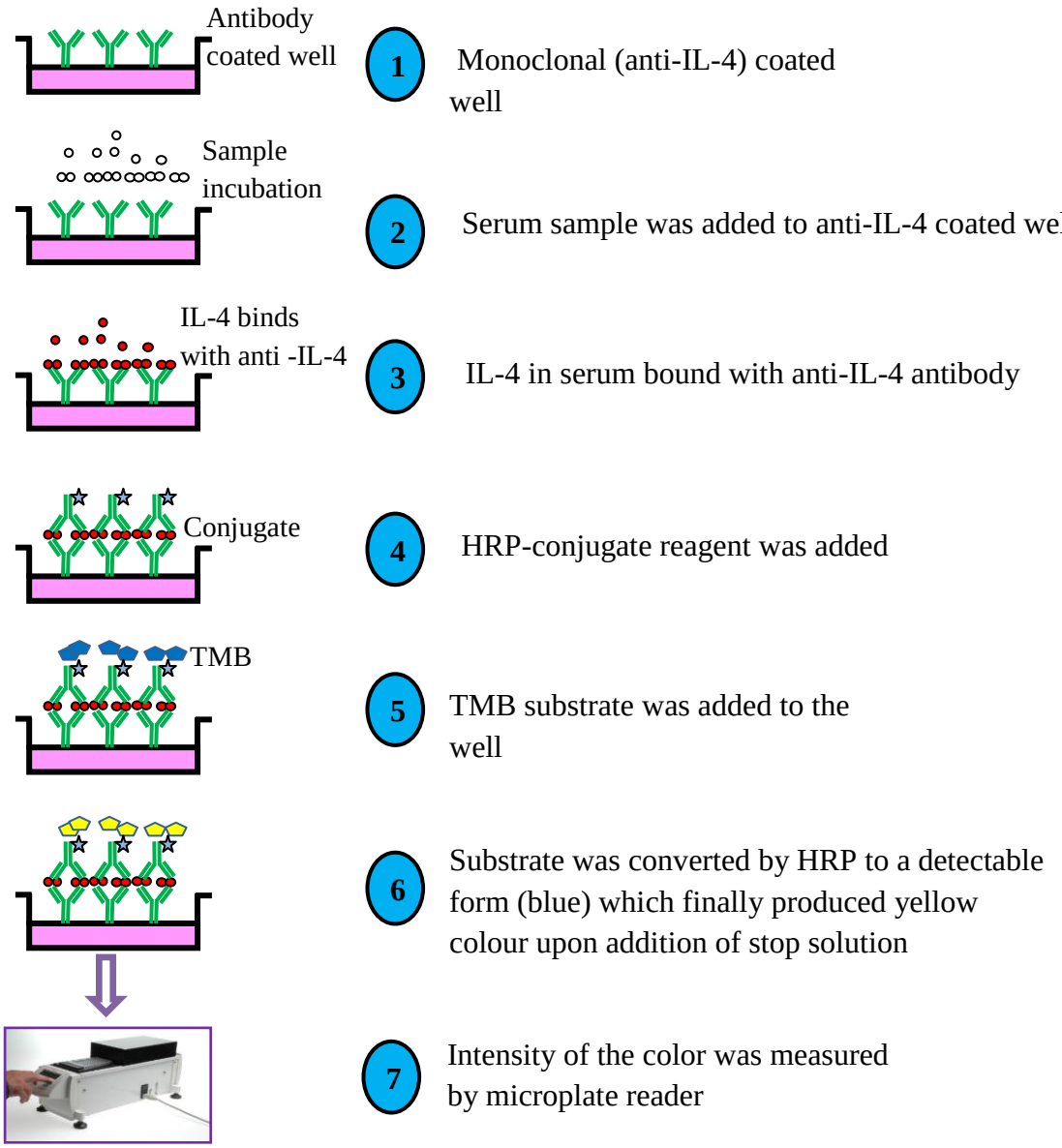

Figure 4.1 Stepwise assay serum IL-4 by IL-4 Elisa kit

4.2.6 Statistical analysis

Statistical analysis for the study was performed using the Statistical Packages for Social Sciences (SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) software. Normality of the distributions of variable was verified by Q-Q plot. Due to the skewed distribution of the raw data, log transformed values of water, hair, nail arsenic, and serum IL-4 levels were used. Spearman correlation coefficient tests were performed to evaluate the correlations of serum IL-4 levels with arsenic exposure (water, hair and nail arsenic concentrations). Multiple regression analyses were performed to assess the effects of age, sex, BMI, smoking, occupation, income and education along with arsenic exposure metrics on serum IL-4 levels. The study subjects in the arsenic-endemic areas were stratified through frequency test into 'low', 'medium' and 'high' exposure groups based on the three concentrations of arsenic in the drinking water, hair, and nails. The study subjects in the non-endemic area were used as a reference group ('lowest' exposure group). In addition, the study subjects were divided into three groups ($\leq$10 μg/L, 10.1-50 μg/L and > 50 μg/L) (based on the regulatory upper limit of water arsenic concentration set by WHO (10 μg μg/L), and the Bangladesh Government (50 μg/L). Finally, dose-response relationships of arsenic exposure metrics with serum IL-4 levels were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. Spearman correlation coefficient tests were performed to evaluate the correlations of serum IL-4 levels with IgE and VCAM-1 levels. A value of $p < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 General characteristics of the study subjects

General characteristics of the study subjects in arsenic-endemic and non-endemic areas were described in the results section of the previous chapter (Chapter 3, Table 3.1). Since we used the same population groups, the demographic or general characteristics of the study populations were also same.

4.3.2 Comparison of serum IL-4 levels between arsenic-endemic and non-endemic populations

Figure 4.2 shows the comparison of serum IL-4 levels in arsenic-endemic and non-endemic populations. It was observed that the serum IL-4 levels (mean ± SE) were significantly higher ($p < 0.05$) in arsenic-endemic (39.45 ± 1.30 pg/ml) population than in non-endemic (33.63 ± 1.81 pg/ml) population.

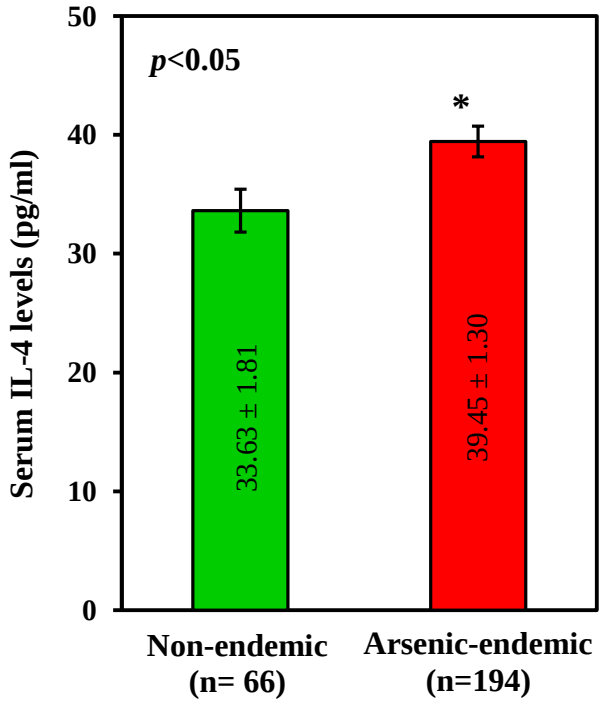

Figure 4.2 Comparison of serum IL-4 levels between arsenic-endemic and non-endemic populations. Green and red columns represent serum IL-4 levels (mean ± SE) of non-endemic and arsenic-endemic populations, respectively. IL-4 levels in arsenic-endemic and non-endemic populations were 39.45 ± 1.30 pg/ml and 33.63 ± 1.81pg/ml, respectively. *Significantly different at $p < 0.05$. p values were from the Independent Sample *T*-test.

4.3.3 Correlation of serum IL-4 levels with arsenic exposure metrics

Figure 4.3 shows the correlations of serum IL-4 levels with drinking water, hair and nails arsenic concentrations of the study subjects. Serum IL-4 levels showed a significant positive (r_s = 0.184, p < 0.01) correlation with arsenic concentrations in drinking water (Figure 4.3A). Serum IL-4 also showed significant positive associations with hair (r_s = 0.204, p < 0.01) and nail (r_s = 0.170, p < 0.01) arsenic concentrations (Figure 4.3B,C).

4.3.4 Multiple regression analyses for the factors associated with serum IL-4 levels

Table 4.2 shows the multiple regression analyses for the factors or variables associated with serum IL-4 levels. In this study, arsenic exposure, age, sex, BMI smoking and socioeconomic conditions (occupation, income and educational status) were considered as possible variables or factors that might influence the serum IL-4 levels. Regression analyses showed that only arsenic exposure (drinking water, hair and nail arsenic concentrations) but not other variables were significantly associated with the elevation of serum IL-4 levels.

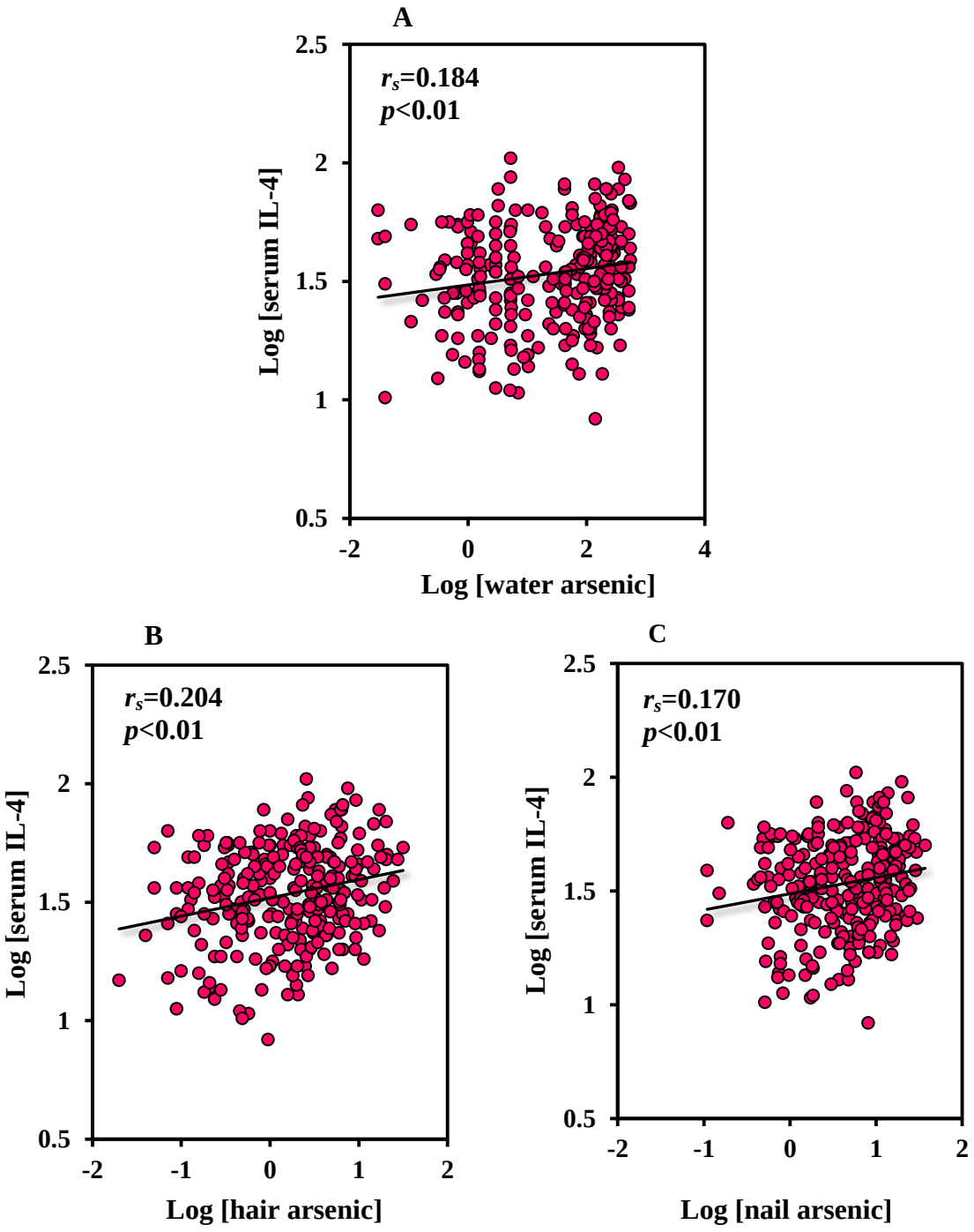

Figure 4.3 Correlation of serum IL-4 levels with arsenic exposure metrics. Correlations of drinking water **(A)**, hair **(B)** and nail **(C)** arsenic concentrations with serum IL-4 levels. Serum IL-4 levels were significantly and positively associated with the arsenic concentrations in drinking water, hair and nails of the study individuals. Log transformed values of arsenic concentrations and serum IL-4 levels were used. r_s and *p*-values were from Spearman correlation coefficient test.

Table 4.2 Multiple regression analyses for the factors associated with serum IL-4 levels

	Dependent variable (Serum IL-4)	
Independent variables	**β-Coefficient**	**p-value**
Water Arsenic	0.034	< 0.01
Age	0.001	0.494
Sex	-0.032	0.358
BMI	-0.004	0.360
Smoking	0.006	0.878
Occupation	-0.001	0.954
Income	-0.002	0.239
Education	-0.006	0.721
Hair arsenic	0.078	< 0.001
Age	0.001	0.505
Sex	-0.035	0.306
BMI	-0.002	0.557
Smoking	0.001	0.981
Occupation	0.000	0.999
Income	-0.002	0.195
Education	-0.008	0.620
Nail arsenic	0.073	< 0.01
Age	0.001	0.458
Sex	-0.036	0.301
BMI	-0.003	0.497
Smoking	0.004	0.905
Occupation	0.000	0.990
Income	-0.002	0.222
Education	-0.009	0.615

Log-transformed values of arsenic concentrations in exposure metrics and serum IL-4 levels were used.

4.3.5 Dose-response relationships of arsenic exposure with serum IL-4 levels

To investigate the dose-response relationship between arsenic exposure and serum IL-4 levels, study subjects were split into four groups (lowest, low, medium and high) based on the arsenic concentrations in drinking water, hair and nails, where the non-endemic study subjects were used as a lowest exposure (reference) group. Intriguingly, serum IL-4 levels were found to be higher in higher exposure gradients compared to the lower exposure gradients of the water, hair and nail arsenic concentrations. Although gradual increases of serum IL-4 levels were observed in the higher concentration gradients compared to the lower gradients of water arsenic, the differences were only significant in the high versus lowest ($p < 0.05$) arsenic exposure groups (Figure 4.4A). Almost similar shape of dose-response relationships of serum IL-4 levels with hair and nail arsenic gradients were observed as it was observed in the water arsenic gradients. In the case of hair arsenic gradients, serum IL-4 levels were significantly higher in the high exposure groups ($p < 0.01$) and medium exposure group ($p < 0.05$) compared to the lowest exposure groups (Figure 4.4B), whereas significantly ($p < 0.05$) higher levels of serum IL-4 were only observed in the high group compared to the lowest exposure group (Figure 4.4C) in nail arsenic concentrations.

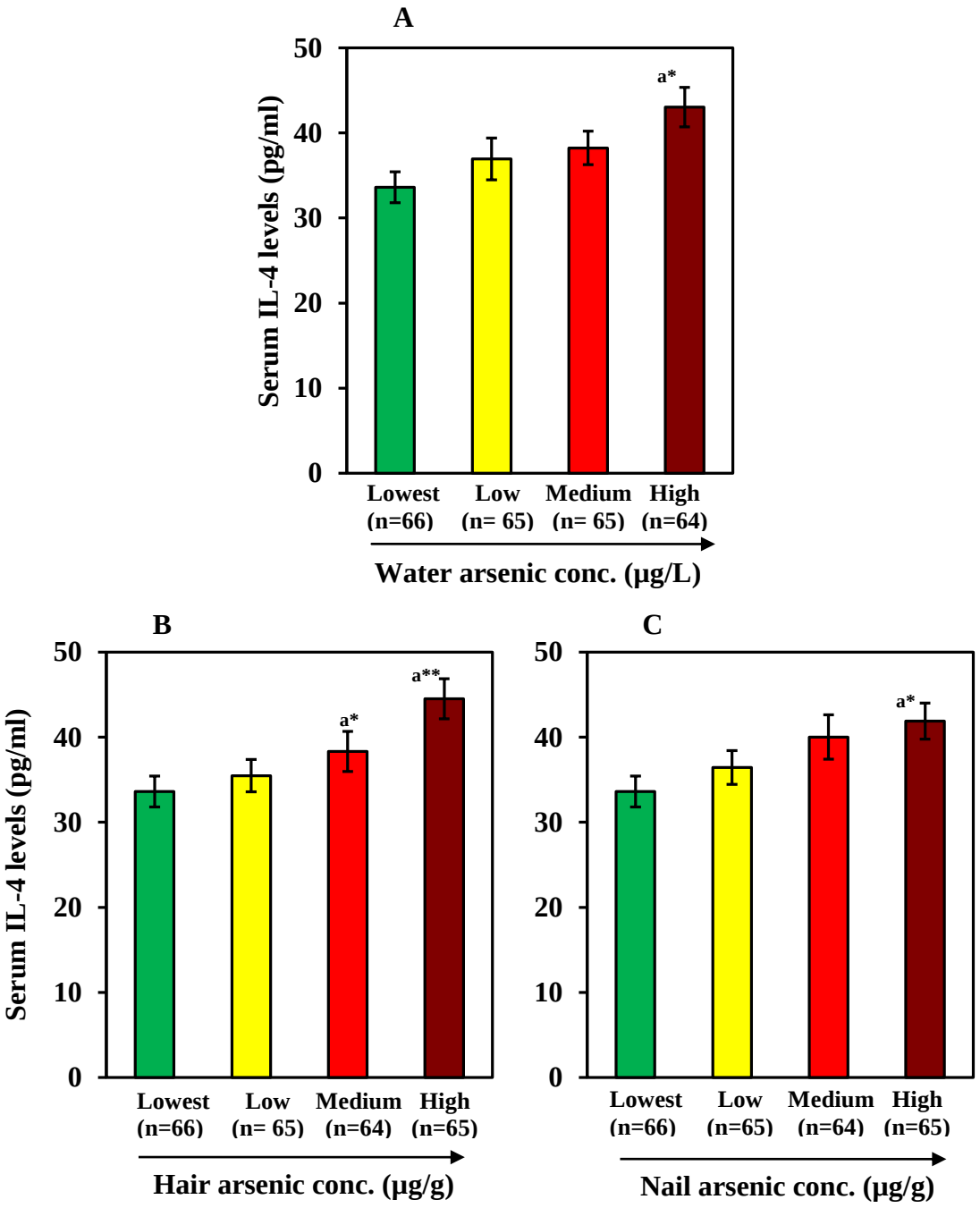

Figure 4.4 Dose-response relationships of arsenic exposure with serum IL-4 levels. Green, yellow, red and dark red columns represent the serum IL-4 levels (mean ± SE) of lowest, low, medium and high exposure groups of study subjects, respectively. The groups based on drinking water arsenic levels were lowest (0.03-10.12 µg/L; n=66), low (0.11- 45.80 µg/L; n=65), medium (45.81-180.00 µg/L; n=65), and high (180.01 – 546 µg/L; n=64). The groups based on hair arsenic levels were lowest (0.02 – 1.00 µg/g; n=66), low (0.05 – 1.96 µg/g; n=65), medium (1.97–4.65 µg/g; n=64), and high (4.66 –31.81 µg/g; n=65). The groups based on nail arsenic levels were lowest (0.15–8.13 µg/g; n=66), low (0.11 – 4.92 µg/g; n=65), medium (4.93 – 11.0 µg/g; n=64), and high (11.01 – 37.42 µg/g; n=65). In the all cases, lowest groups were recognized as the reference group. All *p*-values were from one-way ANOVA. ** $p < 0.01$, * $p < 0.05$; [a] Significantly different from lowest group.

4.3.6 Serum IL-4 levels in the groups based on the regulatory upper limit of drinking water arsenic concentrations

The study subjects were split 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 serum IL-4 levels (Figure 4.5). The serum IL-4 levels (mean ± SE) in the ≤ 10 µg/L, 10.1-50 µg/L and > 50 µg/L groups of study subjects were 34.61 ± 1.61 pg/ml, 35.10 ± 3.10 pg/ml and 41.20 ± 1.57 pg/ml, respectively. Serum IL-4 levels in > 50 µg/L groups were found to be significantly higher ($p < 0.05$) than those in two other groups. Serum IL-4 levels were also found to be slightly higher in 10.1-50 µg/L group as compared to ≤ 10 µg/L group but the difference was not significant statistically.

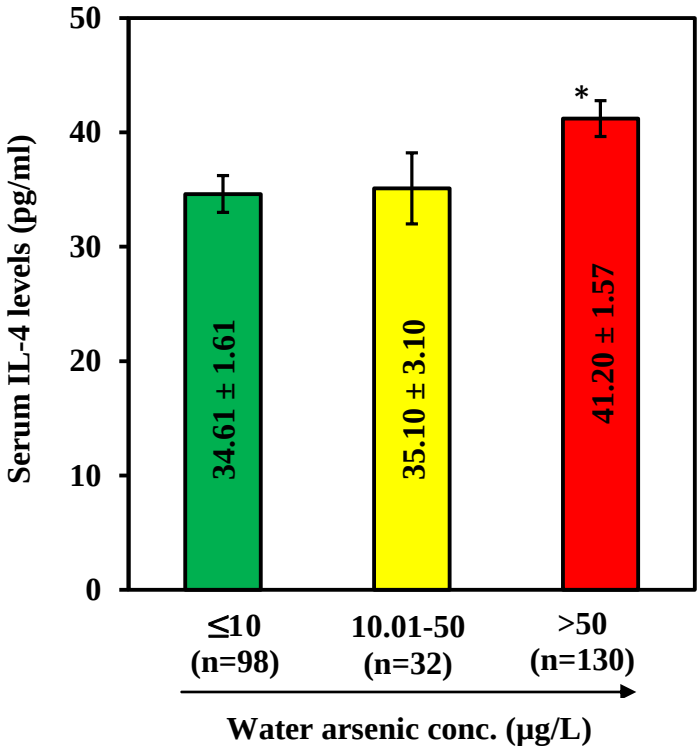

Figure 4.5 Serum IL-4 levels in the three groups based on the regulatory upper limit of drinking water arsenic concentrations. Green, yellow and red bars represent serum IL-4 levels (mean ± SE) of ≤ 10 µg/L (n=98), 10.1-50 µg/L (n=32) and > 50 µg/L (n=130) exposure groups of study subjects. Serum IL-4 levels in ≤10 µg/L, 10.1-50 µg/L and > 50 µg/L groups were 34.61 ± 1.61 pg/ml, 35.10 ± 3.10 pg/ml and 41.20 ± 1.57 pg/ml, respectively. *Statistically significant from ≤10 µg/L at $p < 0.05$.

4.3.7 Correlation between serum IL-4 levels and IgE

Next we examined the correlations of serum IL-4 levels with serum IgE of the study population. Intriguingly we found that serum IL-4 levels showed a significant (r_s = 0.178, p < 0.01) positive correlation with serum IgE levels (Figure 4.6).

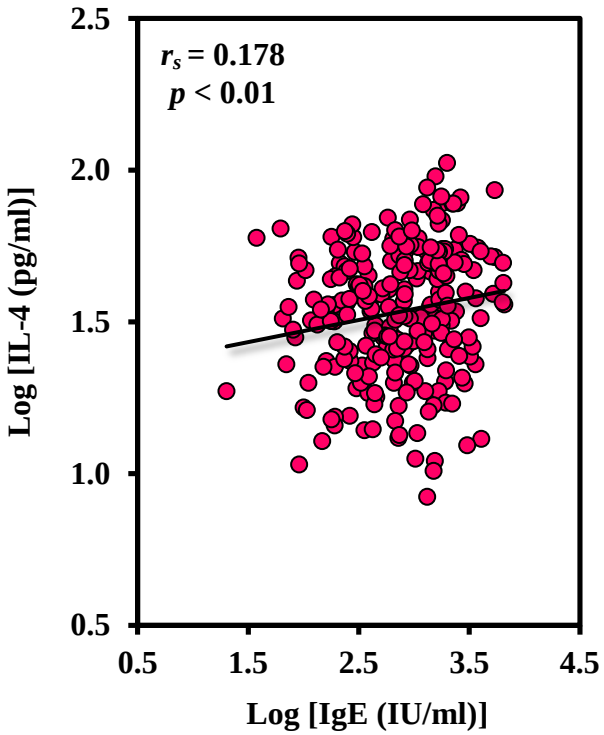

Figure 4.6 Correlations of serum IL-4 levels with serum IgE of the study population. Serum IL-4 levels were significantly and positively associated with serum IgE levels. Log transformed values of serum IL-4 and serum IgE were used. r_s and p-values were from Spearman correlation coefficient test.

4.3.8 Correlations of serum IL-4 levels with circulating VCAM-1 of the study population

In our previous study, we found that arsenic exposure was positively associated with circulating VCAM-1 levels (Karim et al., 2013). We checked the correlation of serum IL-4 levels with circulating (plasma) VCAM-1 among the study subjects who provided blood samples in both the previous and present studies. One hundred seventy eight study subjects in this study were overlapped with our previous study (Karim et al., 2013). Intriguingly we found that serum IL-4 levels showed significant (r_s = 0.237, p < 0.01) positive association with plasma VCAM-1 levels (Figure 4.7).

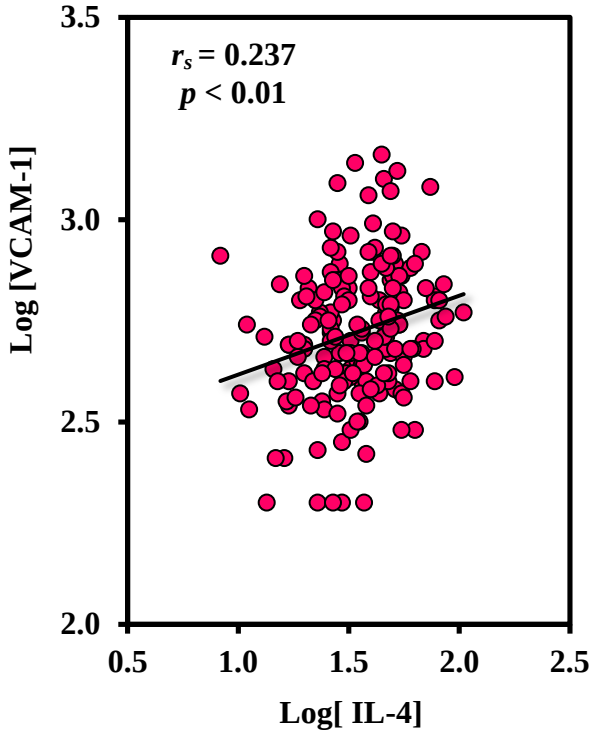

Figure 4.7 Correlations of serum IL-4 levels with circulating VCAM-1 of the study population. Serum IL-4 levels were significantly and positively associated with plasma VCAM-1 levels. Log transformed values of IL-4 and VCAM-1 levels were used. r_s and *p*-values were from Spearman correlation coefficient test.

4.4 Discussion

IL-4 is an important cytokine in the allergic inflammation related to asthma. Chronic exposure to arsenic has been reported to be associated with asthma (Islam et al., 2007; Mazumder et al., 2000; Saha, 1995). In the previous chapter (Chapter-3), we found that chronic exposure to arsenic was implicated in the elevation of serum IgE, a principle component of the allergic reaction leading to asthma. IL-4 has been recognized as a key molecule that regulates the IgE production and allergic inflammation (Platts-Mills, 2001). In this study, we found that serum IL-4 levels were significantly higher in arsenic-endemic population than in non-endemic population (Figure 4.2). We also found the significant positive associations of arsenic exposure metrics (water, hair and nail arsenic concentrations) with serum IL-4 levels (Figure 4.3). Further in dose-response relationship, serum IL-4 levels were found to be significantly higher in the high exposure gradient compared to the lowest exposure gradient (Figure 4.4, 4.5).

To the best of our knowledge, this is the first human study that showed the association between arsenic exposure and serum IL-4 levels. *In vitro* studies showed the contradictory results regarding the effect of arsenic exposure on IL-4. Morzadec et al. (2012) reported that arsenic treatment did not impair IL-4 secretion in human activated T cells, while Cho et al. (2012) reported that arsenic treatment decreased IL-4 production in murine splenocytes. These contradictory results might be due to the variation of the species, types of cells, conditions and doses of arsenic used for the experiments. In the case of metal toxicity, very often results from *in vitro* or animal model experiments cannot be translated to human. This study was particularly important since it, at least in part described the immunoregulatory effect of chronic human exposure to arsenic especially in regard to the asthma.

There are several ways by which IL-4 implicated in asthma. IL-4 induces the ε isotype switching of B lymphocytes into IgE producing B lymphocytes. Because of class switching of B lymphocytes, a large amount of antigen specific IgE are produced that ultimately causes the cross linking of Fcε receptors on mast cells or basophiles though the binding of multivalent antigen. The cross linking of Fcε receptors causes the activation of mast cells and basophiles resulting the histamine

release through degranulation of cells, production of cytokines and lipid mediators. Significant associations of arsenic exposure metrics with serum IgE (Chapter 3) and IL-4, and the significant interrelationship of these two circulating molecules (Figure 4.6) argued that chronic arsenic exposure-related asthma might be mediated though IgE-IL-4 pathways (Figure 4.8). Furthermore, IL-4 has been reported to play an important role in promoting allergic inflammation by inducing expression of vascular cell adhesion molecule-1 (VCAM-1), which directs the migration of T lymphocytes, monocytes, basophils, and especially eosinophils to the site of allergic inflammation (Thornhill et al., 1991). In our previous study, we found that arsenic exposure was positively associated with plasma VCAM-1 levels (Karim et al., 2013). We conducted this study on the same population group, however, the total number of study subjects were different. Elevated levels of IL-4 observed in this study led us to analyze the relationship of serum IL-4 with VCAM-1 among the study subjects who provided blood samples in both the previous and present studies. One hundred seventy eight study subjects in this study were overlapped with our previous study across the serum VCAM-1 levels (Karim et al., 2013). The results showed that IL-4 levels in the overlapping study subjects had a significant positive correlation with plasma VCAM-1 levels (Figure 4.7). This intriguing relationship further strengthened the notion that elevated level of IL-4 observed in this study might be one of the key mediators for arsenic-induced allergic inflammation leading to asthma. IL-4 is a signature cytokine for T_H2 differentiation of $CD4^+$ helper T cells. Differentiation of uncommitted T_H0 $CD4^+$ helper T cells to T_H2 subset induces the secretion IL-4. T_H2 subset differentiated from $CD4^+$ helper T cell is the principal cellular source of IL-4 production. Differentiation of $CD4^+$ helper T cells to T_H2 cells and increased production of IL-4 ultimately inhibit the T_H1 differentiation and production of interferon- γ (IFN-γ), a signature cytokine of T_H1 subset of $CD4^+$ helper T cells (Abbas et al., 1996 ; Mosmann and Sad, 1996). Shifting physiological microenvironment toward T_H2 cell differentiation from T_H1 is an important step for allergic inflammation (Busse and Lemanske, 2001). In this study, we could not show whether and how arsenic exposure exerts its effect on shifting $CD4^+$ helper T cells toward T_H2 differentiation. Therefore, further studies are required in future to depict the effect of arsenic exposure on the regulation of $CD4^+$ helper T cells differentiation.

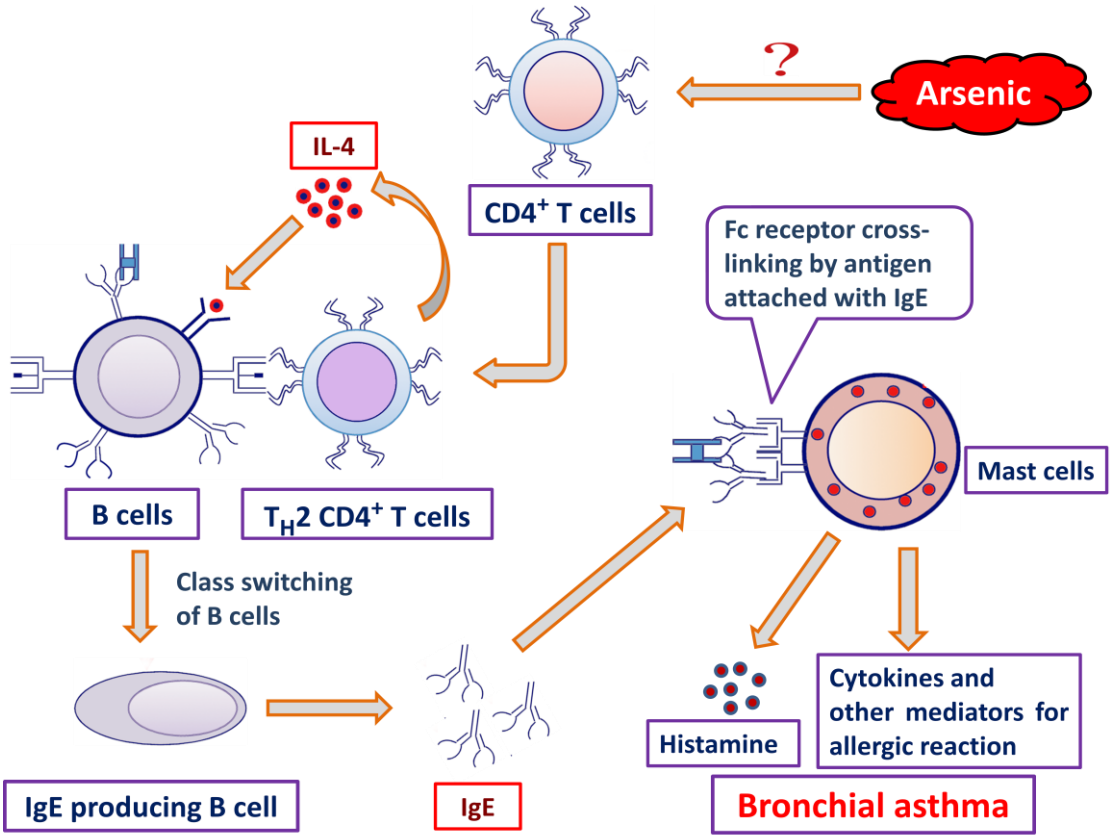

Figure 4.8 Proposed mechanism of arsenic-induced bronchial asthma. Chronic arsenic exposure may cause the elevation of circulating IL-4 levels. However, this study does not clarify how arsenic exposure increases IL-4 production. One possibility is that arsenic exposure stimulates $CD4^+$ T cell to be differentiated into T_H2 subset which is the principal cellular source of IL-4. IL-4 induces isotype switching of antigen-sensitized B lymphocytes into IgE producing B lymphocytes and a large amount of antigen specific IgE is produced. This elevated levels of IgE upon binding of multivalent antigen cause the cross linking of Fcε receptors on mast cells. Cross linking of receptors activates mast cells resulting the degranulation to release histamine, and the secretion of cytokines and other lipid mediators for allergic reactions leading to bronchial asthma.

There are several strengths of the present study. First, all the associations were found across the three kinds of exposure metrics (water, hair and nail arsenic concentrations). Although this is not a cohort study, associations observed across the three kinds of exposure metrics provide the evidence in support of the effect of arsenic exposure on serum IL-4. Second, this study was conducted on a good number study population who had a wide variation of arsenic exposure levels. Wide variation of arsenic exposure levels of the study subjects showed a dose-response relationship between arsenic exposure and serum IL-4 levels. From toxicological view point dose-response relationship is important in understanding the toxic dose of any substance.

In spite of several strengths, there were some limitations of this study that warranted further discussion. First, although in this study, we showed the relationships of arsenic exposure with the major circulating biomarkers of bronchial asthma, we did not show the asthma prevalence rates among the study subjects selected for this study. As a continuation of the current study a research has been planned in order to determine the prevalence rates of asthma in the two population groups recruited for this study. Second, this study was designed to be cross-sectional, but not prospective. A cohort based study is needed in future for the verification of cause-effect relationship between arsenic exposure and serum IL-4 levels. Third, there might be several demographic characteristics and other variables that could affect the serum IL-4 levels. We considered the same variables (age, sex, BMI, smoking, and socioeconomic condition) as we selected in the previous chapter (Chapter 3) for the analysis of the association of arsenic exposure with serum IgE levels. Except arsenic exposure metrics, we did not find any significant association of those variables with serum IL-4 levels (Table 4.2). However, we could not completely ignore the effect of other variables such as co-exposure to other metals, insecticides or pesticides or other genetic and non-genetic factors that could influence the serum IL-4 levels. More extensive study is required in future to check the effects of those variables on serum IL-4 levels. Fourth, as we mentioned in Chapter 3, the study population were lean (mean BMI for non-endemic and endemic population were 21.47±2.72 and 20.91±3.69 respectively). Thus, the results of the current study may not be generalizable to other study populations. Fifth, although based on the results of this study, we demonstrated a hypothesis showing a pathway by which arsenic exposure

might induces asthma (Figure 4.8), we did not provide any *in vivo* data in support of our hypothesis in this study. Therefore, *in vivo* experiments are needed to verify the hypothesis in future.

4.5 Conclusions

This study demonstrated that serum IL-4 levels were significantly higher in arsenic-endemic population than in non-endemic population. Serum IL-4 levels showed significant positive associations with arsenic exposure metrics (water, hair and nail arsenic concentrations). Further, arsenic exposure metrics showed novel dose-response relationships with serum IL-4 levels. Intriguingly, serum IL-4 levels also showed significant positive correlations with circulating IgE and VCAM-1. Thus, the elevated levels of serum IL-4 observed in this study might be involved in the development of allergic reactions leading to bronchial asthma in arsenic-endemic population.

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Chapter 5

SUMMARY OF THE THESIS

CHAPTER 5: SUMMARY OF THE THESIS

5.1 Objectives

Arsenic exposure is a major threat to the public health in many countries of the world including Bangladesh. Arsenic exposure has been reported to be associated with several chronic diseases including asthma. Asthma is largely mediated through allergic reactions involving several molecules. Immunoglobulin E (IgE) and interleukin-4 (IL-4) are the major molecules implicated in allergic asthma. Associations of chronic arsenic exposure with IgE and IL-4 have not yet been documented. Therefore, this study was designed to explore the associations of arsenic exposure with circulating IgE and IL-4 recruiting human individuals from arsenic-endemic and non-endemic areas in Bangladesh.

5.2 Methods

Arsenic-endemic study subjects were selected from the arsenic-contaminated north-west region of Bangladesh. Non-endemic study subjects were selected from Chowkoli, a village under Naogaon District with no history of arsenic exposure. Personal and other relevant information were collected using a standard questionnaire. Local residents (15-60 years of age) were invited to participate in the study. Those who responded spontaneously were asked to convene at a specific location in their village for initial screening purposes in light of the exclusion criteria. Drinking water, hair, nail and blood specimens of the study individuals were collected. Arsenic levels in drinking water, hair and nails were determined by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS). Serum IgE and IL-4 levels were measured by commercially available ELISA kits according to the manufacture's protocol through micro plate reader. Statistical analyses were performed using the statistical Packages for Social Sciences (SPSS) software.

5.3 Summary of results

Serum IgE and IL-4 levels in arsenic-endemic population were significantly different from non-endemic population. The levels (mean ± SE) of serum IgE in arsenic-endemic and non-endemic populations were 1372.55 ± 96.74 IU/ml and 723.08 ±

73.57 IU/ml, respectively, and the levels (mean ± SE) of serum IL-4 in these two populations were 39.45 ± 1.30 pg/ml and 33.63 ± 1.81 pg/ml, respectively. Both serum IgE and IL-4 levels showed significant positive correlations with arsenic concentrations in water, hair and nails. IgE and IL-4 levels were found to be increased gradually in the higher exposure gradients compared to the lower gradients when the study subjects were split into four groups: lowest (reference), low, medium high based on the four concentrations of water, hair and nail arsenic concentrations. Dose-response relationships were also observed among the 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 multiple regression analyses, only arsenic exposure (drinking water, hair and nail arsenic) but not other variables (age, sex, BMI, smoking and socioeconomic conditions) were found to be significantly associated with serum IgE and IL-4 levels. Further, IL-4 levels of the study subjects showed significant ($p < 0.01$ for IgE and $p < 0.01$ for VCAM-1) positive associations with circulating IgE and vascular cell adhesion molecule-1 (VCAM-1). Taken together, the results of this study suggest that elevated levels of serum IgE and IL-4 may be implicated in arsenic-induced bronchial asthma.

5.4 Public health relevance and importance of the study

Arsenic is a potent environmental pollutant that has caused an environmental tragedy in some parts of the world especially in Bangladesh where tens of thousands of people have been affected because of the chronic exposure to arsenic. Contaminated drinking water is the main source of arsenic exposure. However, recent reports suggest that foods are also important source of arsenic exposure since high levels of arsenic were found in the rice and vegetables cultivated in the arsenic-endemic areas. Chronic exposure to arsenic induces a variety of cancers, dermatitis, cardiovascular diseases, diabetes mellitus, liver and kidney dysfunction, peripheral neuropathy and many other complications (Ali et al., 2010; Argos et al., 2010; Chen et al., 2007; Huda et al.,2014; Karim et al., 2013; Mazumder et al., 1998, 2000; Mazumder, 2005; Meliker et al., 2007; Mumford et al., 2007; Tapio and Grosche, 2006; Vahidnia et al., 2008; Wang et al., 2002). Additionally several studies indicate

that arsenic exposure is also associated with respiratory complications including asthma (Islam et al., 2007; Mazumder et al., 2000; Saha, 1995, 2003; Saha et. al., 1999). Asthma is a substantial public health problem among children and adults worldwide and there has been a sharp increase in the global prevalence, morbidity, mortality, and economic burden associated with asthma. According to the Global Initiative for Asthma (GINA), approximately 300 million people worldwide currently have asthma, and its prevalence increases by 50% every decade. Each day 9 Americans die from asthma, this estimates about 3,300 deaths annually (NCHS, CDC, 2001). Prevalences are high (>10%) in developed countries and rates are increasing in developing regions as they become more westernized (Braman, 2006). Worldwide, approximately 180,000 deaths annually are attributable to asthma (Braman, 2003). In an estimate it has shown that 7 million people including 4 million children in Bangladesh suffering from asthma-related symptoms and the prevalence of asthma is 6.9% (Hassan et al., 2002). Therefore, even a small contribution of arsenic exposure on developing asthma may increase thousands of additional asthma patients in Bangladesh or other countries where arsenic is a major threat to the public health. IgE and IL-4 are the main mediators in allergic asthma. Elevated levels of IgE and IL-4 and their significant correlation observed in this study suggest that arsenic exposure-related asthma may be mediated through IL-4-IgE sensitive pathways. Further IL-4 levels were found to be associated with circulating VCAM-1 levels, an important molecule related to the promotion of allergic inflammation. Thus these results shed light on the mechanisms of arsenic-induced asthma. Understanding mechanism is critically important for the prevention and therapeutic intervention of the diseases. The results of the study can provide valuable information for policy makers, physicians and health workers for the formulation of action plan to reduce the health effects of the chronic human exposure to arsenic. Further, the results of this research may be the valuable for the development of awareness against arsenic-induced asthma among the people who are now at risk of arsenic exposure. Therefore, 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.

5.5 Major limitation and recommendation for the future study

Although in this study, we showed the relationships of chronic arsenic exposure with the major circulating biomarkers of bronchial asthma, we did not show the asthma prevalence rates in arsenic-endemic and non-endemic populations. This was one of the major limitations of the present study. As a continuation of the current study a research has been planned in order to determine the prevalence rates of asthma in the two population groups recruited for this study.

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ANNEXURE

Annexure-I

Consent Form (for adults only)

Title: Investigation of association of the arsenic exposure with circulating molecules related to bronchial asthma

I have read the attached information on the research in which I have been asked to participate. I had an opportunity to discuss the details and ask questions about this information. The investigator has explained the nature and purpose of the research and I believe that I understand what is being proposed. I understand that physical examination, samples collections and questionnaires as part of research designed to promote the medical knowledge that has been approved by the the Institute of Biological Sciences of Rajshahii University. I have been informed that the proposed study involving the blood, nail and hair collection have been explained to me together with possible risks involved. I understand that my personal involvement and any particular data from this will remain strictly confidential. Only researcher involved in this study will have access, or where applicable, charity sponsor which funded the research. I understand that when appropriate, my regular doctor will be informed that I have taken part in the study. I understand that I can withdraw my participation in this study in any time and my personal data (including samples) can be excluded from the study if I request the investigators.

I hereby fully and freely consent to participate in the study which has been fully explained to me.

Patient's/Volunteer's Name **Patient's/Volunteer's Signature:** **Date:**

Witness' Name: **Witness' Signature:** **Date:**

As the Investigator responsible for this research or a designated deputy, I confirm that I explained to the patient/volunteer named above the nature and research and purpose of the research to be undertaken.

Investigator's Name: **Investigator's Signature:** **Date:**

Note: *If a participant does not read or speak English the content of this consent form must be translated to the appropriate language.*

Annexure-II

Questionnaires to the patient/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: (if any): ……..
5. Age: ……..
6. Sex: M F
7. Occupation: ……..
8. Body weight: …….. (in Kg)
9. Body height: …….. (in m) …….. (in ft)
10. Blood pressure: ……..
11. Marital status: i) Yes ii) No

12. Members 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 ii) Primary iii) Secondary iv) Higher secondary
    v) Graduate level
15. Housing Status:
    a) Brick with concrete roof (Pakka)
    b) Brick with corrugated tin roof
    c) Mud with corrugated tin roof
    d) Straw (wall) with corrugated tin roof
    e) Others (thatched and tin wall with corrugated tin roof)
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 on arsenic exposure-related health problems

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 or viii) Wife

   ix) Others (specify)……

3. What are the age and sex of children:

   i) 1st child…….. sex: M F

   ii) 2nd child ……..sex: M F

   iii) 3rd child……..sex: M F

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

   ……

5. How long is he/she residing in the study area?

   i) 1 year ii) 2 year iii) 5 years iv) More (specify the year)…….

6. Drinking water sources: i) Tube-Well ii) Kua

   Is the source of drinking water contaminated?

   i) Yes ii) No iii) Not yet confirmed.

   If yes, from when he/she came to know? ……Years

   Has the drinking water source been checked for arsenic contamination?

   i) Yes ii) No iii) unknown

   Has the tube well marked by red color?

   i) Yes ii) No

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

8. Major symptoms of arsenicosis

(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

i) Hair loss: ……

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

k) Hearing Problem: ……

l) Others problems: ……

9. a) Has the subject already gone to the physician?

b) i) Yes ii) No

If yes, for what problem (Specify it)? .....

c) Did the physician give you any medicine?

i) Yes ii) No iii) Unknown

d) What types of medicine (specify the drugs).....

e) Did the physician give you any medicine for the treatment of arsenicosis? i) Yes ii) No

f) What types of medicine (specify the drugs) ……

10. Has any agencies/person checked arsenic levels 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) ……

Thanks for your participation and cooperation.

Name & Signature of the Investigator (s):

Date:

Additional comments: