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IRON STATUS, INFLAMMATION AND ANEMIA IN BANGLADESHI WOMEN EXPOSED TO ARSENIC

A Thesis Presented

by

JOYCELYN M. FARAJ

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

February 2011

Nutrition

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by

JOYCELYN M. FARAJ

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DEDICATION

To my family for giving me their invaluable support.

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I would like to thank my advisor, Dr. Alayne Ronneberg, for her indispensable guidance, support and motivation throughout my academic career.
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I thank all my friends and colleagues who were directly or indirectly involved in the project.

ABSTRACT

IRON STATUS, INFLAMMATION AND ANEMIA
IN BANGLADESHI WOMEN EXPOSED TO ARSENIC

FEBRUARY 2011

JOYCELYN FARAJ, B.S. UNIVERSITY OF MASSACHUSETTS AMHERST

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Iron depletion is the most common nutrient deficiency worldwide and is the leading cause of anemia. The prevalence of anemia in Bangladeshi women has been estimated by others at 45%, but the prevalence of iron depletion (ID) and iron deficiency anemia (IDA) in these women remains unknown. Chronic arsenic (As) exposure, which is a major public health problem in Bangladesh, is associated with increased risk of anemia. Arsenic exposure also triggers inflammatory responses that alter iron parameters, including serum ferritin, rendering IDA assessment more challenging. We assessed the prevalence of ID and IDA in 147 Bangladeshi women, ages 18-33 years, who participated in a larger study of arsenic exposure and skin lesions. The current study includes 75 women with skin lesions (cases) and 72 women without lesions (controls). Blood samples, anthropometric, sociodemographic and dietary data were collected at enrollment. Hb, ferritin and hs-c-reactive protein (CRP) were measured in serum. Overall, the prevalence of anemia (Hb < 120 g/L) was 18%. Although the prevalence of ID (ferritin ≤12mcg/L) did not differ between cases and controls,

the prevalence of anemia was almost three times higher among women with arsenic-related skin lesions compared with controls (25% vs 10%, respectively; $p=0.02$). Of the women with anemia, 27% (N=7) also had ID (Hb<120g/L and ferritin ≤12 mcg/L), indicating IDA. Women with normal iron status had double the concentration of toenail arsenic compared to iron-depleted women (2.9 vs 1.4 μg As/g toenail; $p=0.00$). In addition, the arsenic concentration of their water source was three-times higher than that of iron-depleted women (18.8 vs 6.2 μg As/L; $p=0.03$). Mean CRP was higher in cases than controls ($p=0.04$) as well as in those with serum ferritin >12mcg/L compared to those who were iron deplete ($p=0.02$). In multivariable logistic regression,. the risk of ID was 84% lower in women ages 29-33 compared to women ages 18-22 (OR=0.16, 95% CI=0.04, 0.56); every 1μg increase in toenail As level was associated with a 45% lower d risk of ID (OR=0.55 , 95% CI=0.33, 0.94).The presence of inflammation decreased the odds of being classified as ID by 80% (OR=0.20, 95% CI=0.04, 0.96). Much of the anemia in this cohort appears unrelated to iron deficiency, but could be linked to other nutrients, such as folate and vitamin B_{12}, which are involved in both hematopoiesis and arsenic metabolism. It is possible that arsenic exposure in this cohort compromised folate and vitamin B_{12} status. Because these vitamins are also important during pregnancy, additional studies are needed to assess B-vitamin status in arsenic-exposed women and to determine whether B-vitamin status influences reproductive outcomes in these women.

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CHAPTER 1

INTRODUCTION

Iron deficiency is one of the most common nutrient deficiencies, affecting 2 billion people worldwide. It is also the leading cause of anemia (WHO, 2005), and therefore contributes to disability and death. In developed areas of the world, only about 8% of the population has anemia, but in developing regions, the percentage of anemia averages 36% (Crichton, 2006). Estimates of the national prevalence of anemia in Bangladesh have remained constant at 74% for the past 30 years; this high rate of anemia is a major public health concern for Bangladesh, causing a loss of productivity totaling 1.9% of the national gross domestic product (Ahmed, 2000).

According to the World Health Organization (WHO), approximately 50% of all cases of anemia, defined as hemoglobin concentration less than 120 g/L in non-pregnant adults, can generally be attributed to iron deficiency (WHO, 2005), and the leading cause of iron deficiency is dietary inadequacy. In women of reproductive age, menstrual losses also contribute considerably to iron deficiency. In developing countries, such as Bangladesh, parasitic infections such as malaria and hookworm also contribute to iron deficiency (WHO, 2005). In

addition to iron, deficiencies of other micronutrients, including folate and vitamins B_{12}, B_6, and A also contribute to anemia (WHO, 2005; Ronnenberg et al., 2000).

Iron deficiency and anemia hinder normal human functions in all age groups, reducing work performance (Haas JD, 2001). Maternal iron deficiency can also result in adverse pregnancy outcomes, including preterm delivery and lower birth weight (Allen, 2000; Ronnenberg et al, 2004). Impaired immune responses, gastrointestinal abnormalities, changes in the hair and nails, impaired thermogenesis, altered thyroid metabolism, and changes in catecholamine turnover have also been observed in subjects with iron deficiency (Crichton, 2006).

In addition to its high prevalence of anemia, Bangladesh also faces a major health challenge: chronic arsenic poisoning. An estimated 57 million Bangladeshis are chronically exposed to arsenic via drinking water (British Geological Survey, 2001). The mechanism of arsenic toxicity remains largely unknown; however, there is evidence that arsenic poisoning may influence hematological variables (Hernandez-Zavala et al., 1999; Szymanska-Chabowska et al. 2002; Tchounwou et al, 2003). *In vivo* and *in vitro* studies have shown that arsenic can bind to animal and human hemoglobin (Delnomdedieu et al. 1995; Lu et al, 2004; Winski & Carter, 1995) and can alter heme metabolism, hemoglobin concentration and red blood cell morphology (Delnomdedieu et al, 1994; Flora et al 2005; Kannan et al, 2001). Other studies found that chronic

ingestion of arsenic-contaminated drinking water altered heme metabolism by increasing the activity of two key enzymes in heme metabolism, porphobilinogen deaminase and uroporphyrinogen decarboxylase, in peripheral red blood cells and increasing total urinary excretion of porphyrins (Hernandez-Zavala et al. 1999).

The effects of chronic arsenic toxicity on the heme system and on iron status remain largely unexplored. The main goal of this research project is to study the relationship between biomarkers of iron status, inflammation, anemia, and arsenic-associated skin lesions in women of reproductive age from Bangladesh.

CHAPTER 2

IRON

2.1 Introduction and Biological Functions

Iron (Fe) is an essential nutrient, for numerous biological processes, including electron transfer reactions and substrate oxidation-reduction, regulation of gene expression, binding and transport of oxygen, and regulation of cell growth and differentiation (Beard, 2006)   Although essential, iron can also be a potential toxicant to cells, hence its bioavailability is highly regulated by various complex mechanisms. The regulating mechanisms used by the body include control of dietary iron absorption, iron entry into cells, intracellular storage of iron as ferritin, release of iron from cells, and sequestering of free iron by iron binding proteins.

Iron exists in two oxidation states in aqueous solution, ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}), and it can change from one state to the other by the subtraction or addition of an electron. Certain reducing agents, such as vitamin C (ascorbic acid) can convert ferric iron to ferrous iron. Exposure to oxygen can also convert ferrous iron to its ferric form. This inter-conversion of oxidation states is essential for electron transfer reactions as well as for reversible binding to ligands.

Preferred ligands of iron include oxygen, nitrogen and sulfur atoms (Beard, 2006).

2.1.1 *Iron-Containing Proteins*

Most of the iron in plant or animal cells is stored within large complex proteins such as hemosiderin or ferritin. The remainder of the iron is either contained as an essential functional component within proteins and enzymes, or in iron transport proteins (Beard, 2006). The main categories of iron-containing proteins in mammals include heme-containing hemoproteins (e.g., hemoglobin, myoglobin, and cytochromes); iron-sulfur cluster-containing enzymes (such as flavoproteins); iron storage proteins (e.g., ferritin); iron transport proteins (e.g., transferrin, lactoferrin); and iron-dependent enzymes (Beard 2006).

Heme-proteins: The key function of iron is to move oxygen from the environment to terminal oxidases. Oxygen is bound to iron in the porphyrin ring of the heme moiety in hemoglobin, found within red blood cells (RBC), or in myoglobin, the facilitator of oxygen diffusion in tissues (Beard, 2006). Hemoglobin (Hb, MW 64 KDaltons) is a tetrameric protein with two pairs of identical subunits (alpha-2 and beta-2). Each of the subunits has one heme (iron-protoporphyrin-IX) prosthetic group whose ferrous iron reversibly binds dioxygen. The four subunits of Hb are not covalently attached, but they do react cooperatively with dioxygen, with specific modulation by pH, carbon dioxide pressure, organic phosphatases, and temperature, all of which determine the efficiency of oxygen transfer from the

alveoli capillary interface in the lungs to the red blood cell/capillary tissue interface in peripheral tissues (Beard, 2006). Decreasing pH causes an allosteric effect (Bohr effect) that decreases the binding affinity of heme iron for dioxygen, thus improving the release of oxygen in tissues where the pH is lower and the CO_2 pressure is higher than in arterial blood (Beard, 2006).

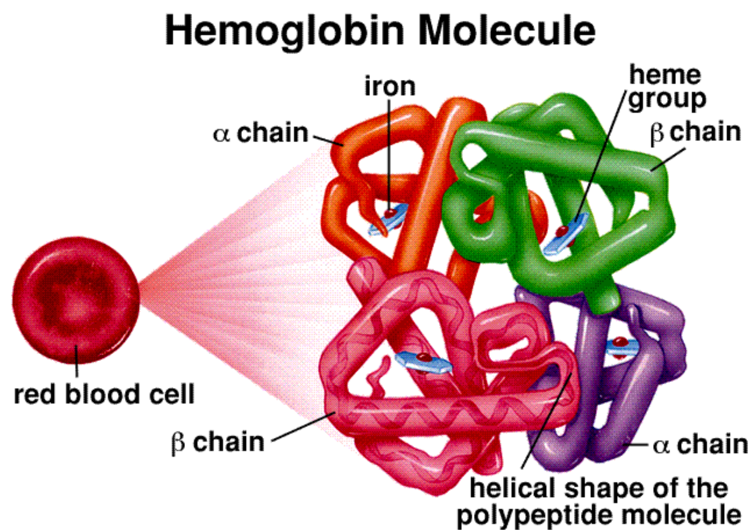


Figure 2.1: Hemoglobin molecule. Iron in heme group binds to the center of the alpha and beta chains of hemoglobin.

Sylvia S. Mader, Inquiry into Life, 8th edition. The McGraw-Hill Companies © 1997

Fe-S Proteins: Another group of iron-containing proteins are iron-sulfur cluster proteins. These proteins contain iron-sulfur centers with equal numbers of iron and sulfide ions (2Fe-2S and 4Fe-4S). They are usually involved in electron transfer reactions, and include proteins of the mitochondrial electron transport chain as well as various mini-electron transport systems (Beard, 2006). Some

Fe-S proteins also have functions in catalysis and biological sensors for iron, oxygen, and superoxide (Flint and Allen, 1996).

Fe Storage Proteins: The proteins involved in iron storage are ferritin and hemosiderin. These proteins play a key role in cellular "housekeeping" in most cells by sequestering free iron, thereby preventing the unwanted effects of iron-catalyzed free radical generation or iron oxidation and precipitation. In addition, these proteins serve as an iron reservoir in specialized cells involved in iron metabolism, such as macrophages of the reticuloendothelial system and parenchymal cells of the liver (Beard, 2006).

The apo-ferritin (iron-free) molecule has a molecular mass of approximately 500 kDaltons, and can hold up to 4,500 iron atoms in a non-toxic, water-soluble, yet bioavailable form (Crichton, 2006). The mechanism of iron release from the ferritin molecules is not well understood, but appears to involve reducing conditions and a more acidic pH (Crichton, 2006). The iron-storage protein ferritin is primarily an intracellular protein; however, minor amounts of ferritin are present in the circulation in proportion to iron stores. Plasma ferritin, unlike intracellular ferritin, is glycosylated and relatively iron-poor. Ferritin is a positive acute-phase reactant, and as such is elevated in states of inflammation.

Ferritin is a soluble protein, but can be degraded to insoluble hemosiderin, which accumulates in lysosomes and also acts as a storage form of iron that is

available for protein and heme synthesis (Ward et al, 1994). Hemosiderin is insoluble and particulate, with a granular appearance when stained, as opposed to ferritin, which is soluble and has a non-granular light blue staining of the hepatocytes or macrophage cytoplasm (Batts, 2007). Increased hemosiderin deposition in the liver and in biliary epithelium is observed mainly in conditions of iron overload (i.e., hereditary hemochromatosis and transfusion-dependent hemoglobinopathies) (Crichton, 2006).

Fe Transport Proteins: Transferrin is the major plasma protein involved in iron transport throughout most of the extracellular fluids of the body, with a continuous circulation from the blood to interstitial fluid. It is a glycoprotein, synthesized mainly in the liver, containing two iron-binding sites, encoded on chromosome 3 (Crichton, 2006). The protein is composed of a single polypeptide chain of approximately 680 amino-acid residues, with two similar amino- and carboxy- terminal lobes, each organized into two distinct domains; each lobe contains an iron-binding site. Transferrin's affinity for Fe^{3+} is high (K_d 10^{-19} to 10^{-20} M). There are six atoms required for iron binding, four of which are provided by the protein (one aspartate, one histidine, and two tyrosine residues) and the remaining two are provided by a carbonate anion, which is essential for iron binding (Crichton, 2006). Conformational changes take place during the binding and release of iron. When the iron-binding site is free, the protein adopts an open conformation, and when iron is bound in the presence of the carbonate

anion, the transferrin iron binding sites take up a pincer-like closed position around the iron atom (Crichton, 2006).

2.2 Iron Absorption

Intestinal iron absorption depends on three conditions: the iron content of the diet, the bioavailability of the dietary iron, and the capacity of the mucosal cells to absorb the iron (Crichton, 2006). There are two kinds of dietary iron: heme and non-heme or inorganic iron. Iron-replete persons will absorb proportionally less of any amount of non-heme iron consumed than will those who are iron-deficient. This type of selective absorption is the main mechanism by which iron is regulated in the human body (Beard, 2006). The recommended dietary intake of iron for adults is around 13-18 mg per day, out of which only 1 mg is absorbed. Even in iron deficiency, absorption is only increased to approximately 2-4 mg/day, and in iron overload, it is reduced to 0.5 mg/day (Miret, 2003).

2.2.1 *Non-Heme Iron Absorption*

Most dietary iron occurs in the non-heme form, which is present in foods as either the reduced ferrous (Fe^{2+}) or the oxidized ferric (Fe^{3+}) form. Non-heme iron is found in both plants and animal sources; in plants, it is present in three major forms: as metalloproteins (plant ferritin), as soluble iron in the sap of xylem, phloem and plant vacuoles, and as nonfunctional iron complexed with plant structural or storage components, primarily in the form of phytates. A large amount of dietary non-heme iron is present as contaminant ferric oxides and

hydroxides. In animal-derived foods, iron can be found in meat products as ferritin and hemosiderin; in egg yolk, it is bound to the phosphoprotein phosphovitin, and in milk it is bound to lactoferrin or associated with fat globule membranes and low molecular weight compounds (such as citrate) (Crichton, 2006).

Upon entering the stomach, non-heme iron is acted upon by gastric juices containing pepsin and hydrochloric acid, which reduce ferric to ferrous iron, making it more bioavailable. Iron is better absorbed in the upper small intestine, mainly in the duodenum, where the low pH enhances its solubility (Miret, 2003). Under normal physiological conditions (i.e. normal pH and presence of oxygen), ferrous iron is quickly oxidized to ferric iron and precipitates as ferric oxyhydroxides; precipitation tends to occur in the luminal contents of the gastrointestinal tract as the pH increases. However, the slightly acidic microclimate in the duodenal surface (pH 6-6.5) helps maintain significant levels of iron in the ferrous form, as does cell surface reductase activity. This microclimate also provides a proton gradient directed toward the cell interior, creating an additional driving force for iron uptake into the enterocyte (O'Riordan et al., 1995).

Non-heme iron is thought to be taken across the brush-border membrane of the enterocyte after being reduced from ferric to ferrous iron by an apical or brush border ferric reductase called duodenal cytochrome b (DCYTB). Once iron is

reduced by ascorbic acid or other reducing agents, ferrous iron can be transported by the divalent metal ion transporter (DMT1), which transports only ferrous iron (Gunshin, Mackenzie et al, 1997). Iron deficiency and hypoxia stimulate duodenal expression of DMT1, DCTYB and ferroportin (an iron exporter), leading to increased iron absorption (Zimmermann and Huller, 2007).

2.2.2 *Heme Iron Absorption*

Hemoglobin and myoglobin from animal foods are the main protein sources for heme iron. Heme iron has a high intrinsic bioavailability and is soluble in an alkali environment. Heme is released from hemoglobin during digestion in the small intestine and is thought to then bind to a specific receptor on the enterocyte, after which it is internalized via endocytosis (Crichton, 2006). Absorbed heme is acted upon by heme oxygenase 1 (HOX1) in the enterocyte to release iron to the soluble cytoplasmic pool (Beard, 2006). Release of iron from heme by HOX1 appears to be the rate-limiting step in heme-iron absorption (Crichton, 2006).

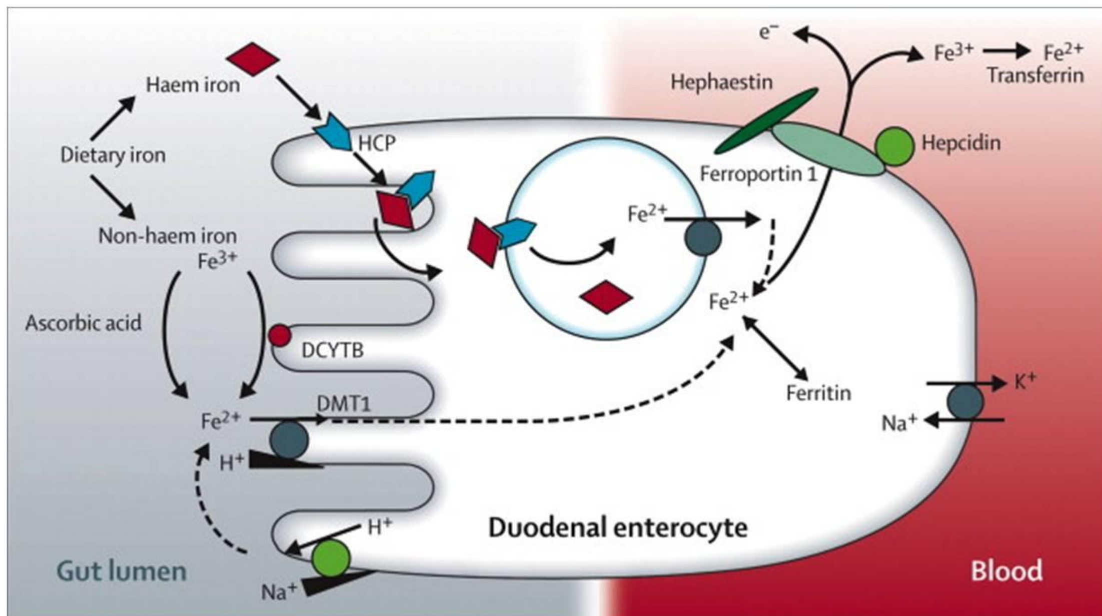


Figure 2.2: Transport of heme and non-heme iron through enterocyte

Zimmermann MB & Hurrell RF. Nutritional Iron Deficiency. *Lancet*. 2007 Aug 11;370(9586):511-20

2.3 Iron Stores and Mobilization

Once inside the enterocyte, iron from both heme and non-heme sources enters a low molecular-weight pool, where it can either be stored in the mucosal cell as ferritin or it can be transported across the basolateral membrane into the plasma by the transmembrane protein ferroportin 1, also known as IREG-1 (Crichton, 2006). This pool of intracellular transit iron is referred to as “labile iron”.

In order for absorbed iron to be incorporated into apotransferrin, it needs to be oxidized to its ferric form either by hephaestin (a membrane bound protein) or by ceruloplasmin (the main copper-containing protein in the serum) (Crichton,

2006). The main route of iron delivery to cells is mediated by the transferrin receptor on the cell surface, whose concentration is directly influenced by cellular iron status. The delivery of iron to cells via transferrin is dependent on the expression of transferrin receptors, which have a high affinity for saturated transferrin (Crichton, 2006).

Regulation of transferrin receptor synthesis is mediated by iron-response proteins (IRPs) binding to mRNA iron- response elements (IREs) (Casey, Hentze et al, 1988). As the cellular pool of low-molecular-weight iron decreases, there is an up-regulation of iron uptake into cells, and a down-regulation of the synthesis of iron storage proteins; this mechanism is exerted via the action of iron regulatory proteins (IRP-1, IRP-2) that regulate transferrin receptor and ferritin mRNA translation (Beard, 2006).

2.4 Iron Homeostasis

The mean serum iron level is approximately 20 micromol/L, and the normal plasma transferrin concentration is approximately 30 micromol/L. Each transferrin has two slots for iron; consequently, transferrin is normally about a third saturated with iron (Crichton, 2006). Plasma transferrin typically carries about 3 mg of iron. A decline in the iron storage pools to 15% saturation of iron binding sites results in less than adequate iron being delivered to essential body iron proteins (Beard, 2006).

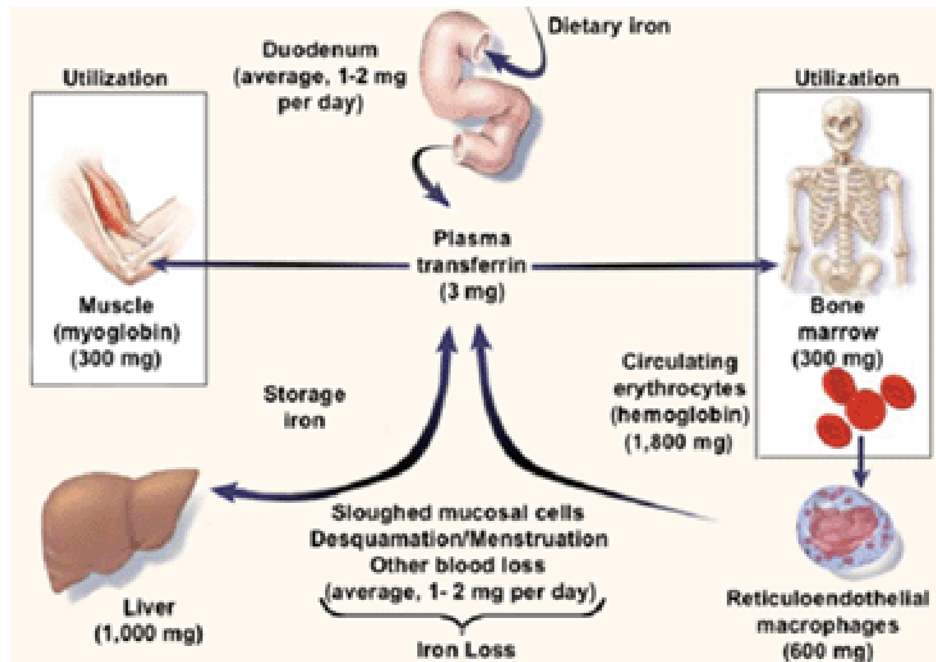


Figure 2.3: Iron stores and mobilization throughout the body.

Andrews NC. Disorders of iron metabolism. *N Engl J Med.* 1999;341(26):1986-95.

The major pathway of iron turnover involves the release of iron from the hemoglobin of destroyed erythrocytes, followed by degradation of myoglobin and iron-containing enzymes (Sherwood et al, 1998). About 85-90% of the body iron stores are found in the erythroid mass. Red blood cells, which have an average lifetime of 120 days, contain about 80% of the body's functional iron (Crichton, 2006). The formation of RBC requires about 30 mg of iron daily, which is balanced by an equal flux of iron from the breakdown of senescent red blood cells by the reticuloendothelial cells in the spleen and Kupffer cells (Miret, 2003). About 85% of iron derived from hemoglobin degradation is re-released to the body in the form of iron bound to transferrin or ferritin (Crichton, 2006).

In the erythroid iron cycle, senescent red blood cells are broken down mainly by macrophages in the spleen, and the extracted iron is returned to the circulation where it binds to transferrin. Plasma transferrin then binds to specific transferrin receptors (TfRs) on surface of cells. The number of transferrin receptors on a cell's surface reflects the cell's iron requirements; the cells that require the most iron are the nucleated red cell precursors in the bone marrow that synthesize hemoglobin: these have the greatest number of receptors (Baker, 1994). Transferrin binds to the receptors on these erythroid precursors in the bone marrow, and the cycle is completed when new RBCs enter the circulation in the following 7-10 days. Iron absorption increases during enhanced erythropoietic activity. The rate of erythropoiesis is regulated by the concentration of erythropoietin, produced by the kidneys (Crichton, 2006). Iron deficiency increases iron transfer through the cycle to by stimulating increased ferroportin expression on macrophages, hepatic synthesis of transferrin, and expression of transferrin receptor (TfR1) in the bone marrow and other tissues (Zimmermann and Huller, 2007).

Two essential regulators of iron absorption recently identified are hepcidin, an antimicrobial peptide synthesized by the liver (Ganz, 2003), and hemojuvelin, a 426-residue protein that shows homology to a molecule involved in axonal guidance in the central nervous system (Papanikolaou et al., 2004). Hepcidin is an antimicrobial peptide that was shown to be involved in regulation of iron

homeostasis in animal studies (Nicolas et al., 2001; Pigeon et al., 2001). The mRNA for murine hepcidin appears to be upregulated during parenteral or dietary iron overload as well as during immune stimuli (treatment with lipopolysaccharide) (Crichton, 2006). Hepcidin is a negative regulator of iron uptake in the small intestine and of iron release from macrophages (Crichton, 2006). In the absence of hepcidin, there is both increased absorption of dietary iron, leading to iron overload, and uncontrolled release of iron sequestered by macrophages, resulting in splenic iron deficiency (Crichton, 2006). In vitro studies have shown that interleukin-6 (IL-6) induces hepcidin during inflammation (Nemeth et al., 2004). Plasma hepcidin binds ferroportin and blocks iron uptake from the duodenum and prevents iron release from macrophages (Crichton, 2006). Not much is known about how hemojuvelin functions, but it appears its action involves the regulation of hepcidin expression (Crichton, 2006).

Body iron losses are relatively small (1-2 mg per day), consisting of losses via epithelial cells (skin, gastrointestinal cells, urinary tract cells) and fluids (tears, sweat, and menstrual losses) (Miret, 2003). These iron losses are distributed among gastrointestinal tract, skin, and urinary tract in a ratio of 6:3:1 (Crichton, 2006). For a 55-kg, non-menstruating woman, the basal iron loss would equal 0.77 mg/day. Menstruation increases the amount of iron loss, and absorption of 1.36 mg of iron is the median requirement for maintenance of iron balance in normal menstruating women. To maintain balance in 95% of women, absorption of 2.84 mg/day of iron is required (Crichton, 2006).

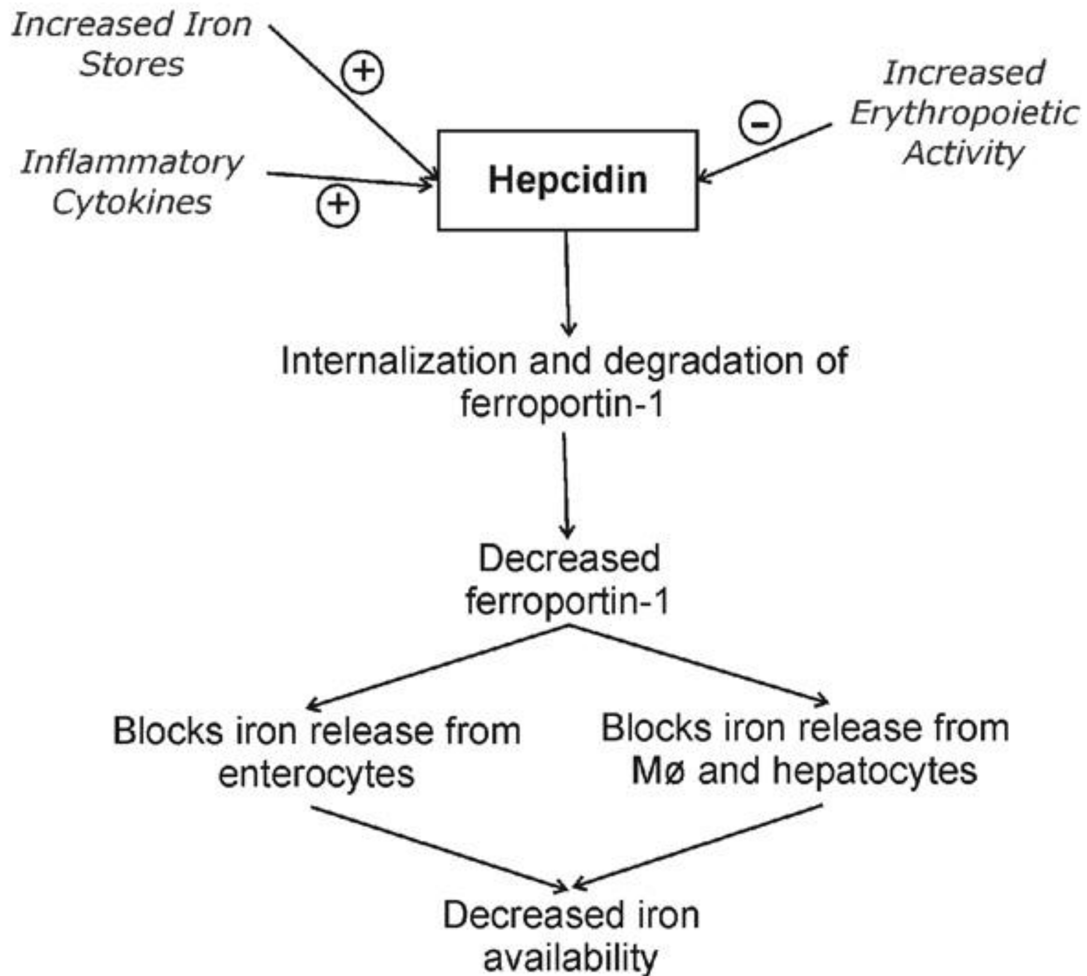


Figure 2.4: Effects of hepcidin on iron absorption and iron release from macrophages.
Cornell University. http://diaglab.vet.cornell.edu/clinpath/modules/chem/femetb.htm

The liver contains about 60% of the body's ferritin; the remaining 40% is found in muscle tissues and cells (macrophages) of the reticuloendothelial system (Crichton, 2006). Theoretically, up to 4500 ferric iron atoms can be stored in one ferritin protein, and in-vivo, ferritin is normally about 20% saturated (800 iron sites occupied) (Beard, 2006). The amount of ferritin synthesized by cells is under the

control of the mRNA-binding protein IRP, which binds with high affinity to an IRE located in the 5'-untranslated end of ferritin mRNA. A similar set of IREs exists on the 3' end of the mRNA for transferrin receptor and DMT-1 that allows reciprocal regulation of iron storage and iron uptake (Crichton, 2006). In low iron situations, IRP1 binds to the IREs of various iron proteins to regulate the translation of the mRNA transcripts by stabilizing the mRNA and allowing translation of the peptide (Worwood, 2002). This results in an increase in the availability of erythrocyte free iron. The concentration of serum transferrin increases, but there is decreased saturation. In the presence of adequate cellular iron, binding of iron by IRP changes the conformation of the protein and prevents it from binding to the mRNA. The mRNA is quickly degraded and synthesis of transferrin receptors is reduced (Worwood, 2002).

2.5 Iron Deficiency

Iron deficiency is characterized by exhausted iron stores, which occurs when serum ferritin concentration drops below 12 µg/L. Simultaneous anemia (Hb<120 g/L) leads to iron deficiency anemia. Clinical symptoms of iron deficiency include the signs and symptoms of anemia (tiredness and lassitude). Clinical manifestations include glossitis, angular stomatitis, koilonchya (spooning of the nails), blue sclera, esophageal webbing, and microcytic hypochromic anemia (Beard, 2006). Impaired immune function and responses, gastrointestinal abnormalities, changes in hair and nails, impaired thermogenesis and muscle function, altered thyroid metabolism, and changes in catecholamine turnover are

symptoms observed in patients with iron deficiency (Beard, 2006; Crichton, 2006). Iron deficiency also increases the risk of adverse pregnancy outcomes and impaired infant development (Zimmermann & Hurrell, 2007). Iron deficiency is normally treated with an oral administration of 125 to 250 mg/day of ferrous sulfate, which should deliver 39 to 72 mg of elemental iron per day and will normally return a deficient person to normal iron levels within 12 weeks (Beard, 2006).

2.6 Assessment of Iron Status

The best minimally invasive method of assessing iron status is measurement of serum ferritin concentration, which bears a quantitative relationship to iron stores in the range of 20 to 200 µg/L, with each 1 µg/L indicative of 8 mg of storage iron (Crichton, 2006). A serum ferritin concentration of 12 µg/L indicates exhaustion of iron stores, and any values below this indicate depletion of the functional iron compartment. Iron stores can also be invasively assessed by measuring the iron content of bone marrow, through liver biopsies or by quantitative phlebotomy. The gold standard for iron store estimation is staining a bone marrow aspirate for iron.

Despite its usefulness in assessing iron status, ferritin is an acute-phase reactant, and as such it is elevated in states of infection, inflammation, neoplasia, hepatic dysfunction and alcohol consumption, resulting in misleadingly high serum ferritin concentration (Crichton, 2006). During an acute phase response,

acute phase proteins (APP) are produced prior to the full activation of the immune response to prevent tissue damage and to remove harmful molecules and pathogens. Increased production of APP is due to changes in their production by hepatocytes, which are in turn regulated by cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor alpha (Feelders et al, 1998). The APPs that are increased during inflammation include: C-reactive protein (CRP), alpha-1-antichymotrypsin (ACT), alpha-1-acid glycoprotein (AGP), serum amyloid A (SAA), fibrinogen, haptoglobin, ceruloplasmin and ferritin (Worwood, 2007).

The major diagnostic challenge in the assessment of iron status is to differentiate between iron deficiency anemia in otherwise healthy individuals and anemia of chronic disease. This is because inflammatory disorders tend to increase circulating hepcidin concentrations, and hepcidin blocks iron release from enterocytes and the reticuloendothelial system, resulting in iron-deficient erythropoiesis (Zimmermann & Hurrell, 2007). The distinction between anemia of chronic disease and iron deficiency anemia is also difficult because increased serum ferritin in anemia does not rule out iron deficiency anemia in the presence of inflammation. A commonly used marker of inflammation is C-reactive protein (CRP). However, the extent of increase of CRP concentration that invalidates the use of serum ferritin to diagnose iron deficiency is unclear, although CRP values higher than 10-30 mg/L have been used (Zimmermann & Hurrell, 2007). In our study we will use an hs-CRP cut-off value of 3mg/L to indicate inflammation.

Moreover, the increase in CRP during the acute-phase response is typically shorter in length than the increase in serum ferritin. Alternative markers of inflammation include the alpha-1-acid glycoprotein (AGP) because it tends to increase later during infection than CRP and remains elevated for several weeks (Wieringa, 2002).

Measurement of the soluble transferrin receptor (sTfR) in plasma may aid in distinguishing iron deficiency anemia from anemia of chronic disease. This is because the main determinants of plasma sTfR are the erythroid mass in the bone marrow and iron status. sTfR is derived mostly from developing RBCs and hence reflects the intensity of erythropoiesis and the demand for iron; the concentration rises in iron deficiency anemia and it is a marker of the severity of iron deficiency only when stores have been exhausted and erythropoiesis is increased, provided that there are no other causes of enhanced erythropoiesis (WHO, 2007; Cook, 2005; Skikne et al, 1990). Also, plasma sTfR is not particularly affected by the acute-phase response, but it might be affected by malaria, age, and ethnicity (Cook, 2005; Verhoef et al, 2001; Menendez et al, 2001)

In developing countries, where there is a high frequency of infection, iron status assessment should include measures of plasma ferritin, whole blood hemoglobin as well as plasma sTfR (Mei, 2005). When possible, additional measures such as whole blood zinc protoporphyrin (ZPP), plasma CRP, and/or AGP should also

be completed (Zimmermann, 2005). In an anemic individual with high CRP, AGP, or both, high sTfR and ZPP concentrations are probably indicators of concurrent iron deficiency, despite high ferritin (Zimmermann and Hurrell, 2007). The WHO recommends that iron assessment include hemoglobin, serum ferritin and transferrin receptor, as well as at least one acute phase protein, with CRP, ACT and AGP as first choices (WHO, 2007).

CHAPTER 3

IRON DEFICIENCY AS A PUBLIC HEALTH PROBLEM

Iron deficiency is the most prevalent single nutrient deficiency in the world and is recognized by the World Health Organization (WHO) as 1 of the 10 greatest global health risks in existence today (WHO, 2002). Iron deficiency is considered the primary cause of anemia, hence iron-deficiency anemia (IDA) and anemia are often used synonymously, and prevalence of anemia has often been used as proxy for IDA (WHO, 2008). The WHO estimates the number of persons with anemia worldwide at almost 2 billion, with approximately 50% of the cases attributable to iron deficiency. However, the prevalence varies according to population groups and local conditions (WHO/UNICEF/UNU, 2001). Despite the fact that iron deficiency is considered the primary cause of anemia, there are not enough data on the prevalence of iron deficiency, possibly due to the difficulty in assessing iron status with accuracy using a single indicator. Therefore, in order to acquire reliable information on existing iron deficiency, a combination of biomarkers must be used (WHO, 2008).

Anemia is seldom present in isolation. It usually coexists with numerous other conditions, such as malaria, parasitic infections, nutritional deficiencies, and

hemoglobinopathies. Risk factors for developing anemia include: low intake of iron, poor absorption of dietary iron from diets high in phytates or phenolic compounds (especially during periods of the life cycle when iron requirements are high), heavy blood loss (menstruation, delivery, or internal bleeding), parasitic infection (hookworm, ascaris, schistosomiasis), acute and chronic infection (malaria, cancer, tuberculosis, HIV), presence of other micronutrient deficiencies (vitamin A, vitamin B_{12}, folate, riboflavin), and the impact of hemoglobinopathies (WHO, 2008).

3.1 Epidemiology of Iron Deficiency

Iron deficiency is a global public health problem, affecting both developed and developing countries with major consequences for human health as well as social and economic development. It occurs in all stages of the life cycle, but the groups more susceptible to iron deficiency and anemia are preschool children, reproductive-aged women, and pregnant women. According to the WHO, from a global perspective, the prevalence of anemia is highest in pre-school aged-children (47.4%) and lowest in men (12.7%); however, the highest number of individuals affected is non-pregnant women (468.4 million) (WHO, 2008). The prevalence and severity of iron deficiency is considerably greater in women than in men due to the physiologic requirements related to reproduction, such as menstruation, pregnancy, and lactation (Islam, 2001).

The overall prevalence of anemia among women in developing countries is 42%, whereas in developed countries, the prevalence remains under 10% (WHO

2008). Women are affected the most by anemia in South East Asia, where 200 million women are anemic. Of these, 182 million are non-pregnant women of reproductive age. In Bangladesh, the estimated prevalence of anemia in 2001 in non-pregnant, non-lactating women from rural areas was approximately 33.2% (taken from random sampling); this translates to more than 11,000 women (WHO, 2008).

3.2 Etiology of Iron Deficiency

Iron deficiency can result from one or more of the following factors: inadequate dietary iron intake, poor absorption, and increased blood losses (Ma, 2007; Beard, 2006). Populations who have increased physiologic iron demands (i.e.: pregnant women and growing children) are at increased risk of not meeting their adequate iron intake. Diets high in phytates or phenolic compounds can inhibit iron absorption due to their ability to chelate and precipitate minerals (Ma, 2007). Increased blood losses, especially in menstruation and delivery for women, as well as gastrointestinal bleeding caused by parasitic infection such as hookworm and malaria can lead to iron depletion. Other nutrient deficiencies, such as vitamin A, folate, or vitamin B_{12}, can lead to anemia.

3.3 Iron Deficiency and Reproductive Health

Iron deficiency and anemia can have a vast number of detrimental health effects, especially in at-risk populations, including preschool-aged children, pregnant women, and women of reproductive age (15-45.99 years). Women of

reproductive age naturally have increased iron needs that are related to menstruation, pregnancy and lactation. Menstrual iron loss, estimated from an average blood loss of 33 mL/month, equals 1.5 mg of iron per day but may be as high as 2.1 mg per day (Cole et al. 1971). There is also a higher demand for iron during pregnancy due to the increase in maternal red blood cell mass, growing fetal requirements (Allen, 1997) and to the compensation for blood losses during delivery. Some evidence suggests that pre-conception iron deficiency can influence the outcome of pregnancy. Mild and moderate anemia at pre-conception have been associated with reduced fetal growth as well as increased risk of adverse pregnancy outcome in a cohort of Chinese women (Ronnenberg et al, 2004).

Iron deficiency anemia during pregnancy is a risk factor for preterm delivery, subsequent low birth weight, and possibly inferior neonatal health (Allen, 2000). A direct causal relationship has not been established for the mechanism leading to these negative health outcomes, but it has been hypothesized that the resulting hypoxia from low iron status and anemia play a role in increasing stress hormones, norepinephrine, and cortisol during pregnancy, which can induce preterm labor (Allen, 2001).

CHAPTER 4

ARSENIC

Inorganic arsenic is a well- established human carcinogen that is associated with a myriad of adverse health effects in addition to cancer (National Research Council, 2001). In the past decades, high levels of the element arsenic have been found in several water supplies in Southeast and Southwest Asia, South and Central America, and some areas in Africa. Millions of persons are exposed globally through arsenic-contaminated drinking water. Light is being shed on the devastating effects of chronic arsenic poisoning as the signs and symptoms of arsenicosis are appearing. There is also increasing evidence that susceptibility to the toxic effects may vary considerably among individuals and can depend on the concentration as well as duration of exposure.

4.1 Arsenic: Properties

Arsenic (As) is a widely-distributed metalloid found mainly in the earth's crust at an average concentration of 2 mg/kg (WHO, 2001). It is usually present in trace amounts in all rock, soil, water, air, and biological tissues. Arsenic is present in over 200 mineral species, the most common of which is arsenopyrite or ferrous arsenic sulphide ($FeAsS_2$); this form has been identified as the prime source of

arsenic pollution in Bangladesh (Fazal et al, 2001a). Arsenic exists as inorganic and organic species. It can be present in four electron valence states: -3, 0, +3 and +5. Inorganic arsenic has two main oxidation states: trivalent [arsenite, As(III)] and pentavalent [As(V), arsenate]. Arsenite is 60 times more toxic than arsenate (Fazal et al, 2001b).

4.2 Arsenic Contamination

Arsenic levels in groundwater average 1-2 micrograms/liter except in areas with volcanic rock and sulfide mineral deposits where arsenic levels can range up to 3mg/liter (WHO, 2001). Terrestrial plants may accumulate arsenic by root uptake from the soil or by adsorption of airborne arsenic deposited on the leaves (WHO, 2001). Non-occupational human exposure to arsenic from the environment is mainly via the ingestion of food and water. In the case of Bangladesh, the population is mainly exposed through the intake of contaminated groundwater, which is used as drinking water; this arsenic is of geological origin. The WHO standard of arsenic in drinking water is 10μg/L, whereas the Bangladeshi standard for drinking water is much higher (50 μg/L) (Smith et al, 2000). The World Health Organization's provisional tolerable daily intake (PTDI) for arsenic is 2.1 μg As/kg-day (Kile et al 2007).

4.3 Arsenic Metabolism

Pentavalent and trivalent soluble arsenic compounds are extensively and rapidly absorbed in the gastrointestinal tract (WHO, 2001). Arsenic metabolism is

usually characterized by two main types of reactions: (1) reduction reactions of pentavalent to trivalent arsenic, and (2) oxidative methylation reactions in which trivalent forms of arsenic are sequentially methylated to form mono-, di-, and trimethylated products with S-adenosyl methionine (SAM) as methyl donor and glutathione (GSH) as an essential co-factor (WHO,2001). The methylation of inorganic arsenic facilitates its excretion in the form of dimethylarsinate (DMA(V)) and methylarsonate (MA(V)), which are readily excreted in urine (WHO, 2001; Vahter, 2002). The trivalent arsenic compounds have been shown to be highly reactive and toxic (Petrick et al.,2000, Vega et al., 2001).

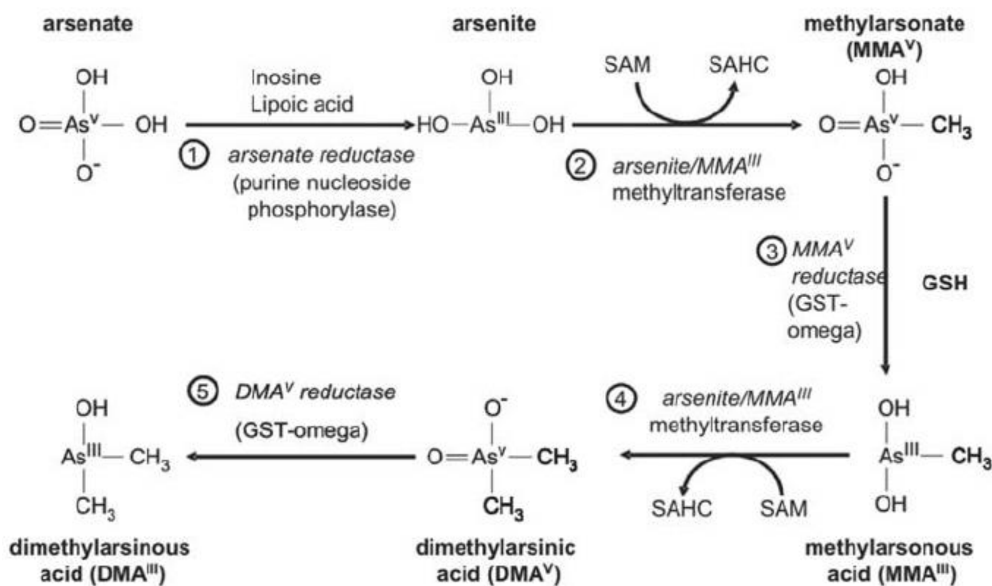


Figure 4.1: Metabolic pathway of arsenic metabolism in vertebrates. Abbreviations: GSH, glutathione; GST-omega, glutathione S-transferase omega; SAHC, S-ademosylhomocysteine; SAM, S-adenosylmethionine. Reproduced from Aposhian HV, Zakharyan RA, Avram MD, Sampayo-Reyes A, Wollenberg ML. 2004. A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxification of the trivalent arsenic species. *Toxicol Appl Pharcamol* 198(3):327-335.

Inorganic arsenic metabolism varies widely among species, populations and individuals. Several factors appear to influence the metabolism of arsenic. Some studies suggest that genetic polymorphisms may have the strongest

influence on rate and efficiency of inorganic arsenic methylation (Lindberg, 2008). Previous studies have shown an association between malnutrition (particularly a protein-deficient diet) and higher risk for several arsenic-related toxic effects (Vahter et al, 1987), possibly due to alterations in arsenic metabolism or reduced antioxidant activity (Milton et al, 2004; Mitra et al, 2004). Several other nutritional factors also influence arsenic methylation, such as availability of methyl groups and micronutrients involved in one-carbon metabolism (Kile & Ronnenberg, 2008; Gamble et al, 2005). Gender, age and smoking have also been shown to influence inorganic arsenic metabolism (Gamble et al., 2005; Loffredo et al., 2003; Steinmaus et al., 2005; Vahter, 2002). Lindberg suggests that sex hormones may enhance the efficiency of arsenic methylation in women of childbearing age (Lindberg et al, 2008).

4.4 Arsenic Toxicity and Carcinogenicity

Arsenic has the ability to impair cellular respiration by inhibiting various mitochondrial enzymes and uncoupling oxidative phosphorylation (Tchounwou, 2003). Most arsenic toxicity results from its interaction with sulfhydryl groups of proteins and enzymes and its substitution for phosphorus in several biochemical reactions (Goyer, 1996). In vitro, arsenic inhibits oxidation of pyruvate and beta-oxidation of fatty acids by reacting with protein sulfhydryl groups to inactivate enzymes, such as dihydrolipoyl dehydrogenase and thiolase (Belton, 1985). Genotoxicity tests suggest that arsenic inhibits DNA repair and induces chromosomal aberrations, sister-chromatid exchanges, and micronuclei

formation in both human and rodent cell cultures as well as in cells from exposed humans (Tchounwou, 2003). Based on single-cell gel electrophoresis experiments (comet assay), arsenic trioxide induces DNA damage in human lymphocytes (Schaumloffel and Gebel, 1998). In other studies, arsenic compounds have been noted to induce gene amplification, arrest cells in mitosis, inhibit DNA repair, and induce expression of the *c-fos* gene and the oxidative stress protein heme oxygenase in mammalian cells (Gonsebatt, 1997; Nakamuro, 1981; Natarajan, 1996; Ramirez 1997).

As inorganic arsenic is metabolized, it is first methylated to (MMA(V)) and (DMA(V)). Methylation of inorganic arsenic involves a two-electron reduction of pentavalent to trivalent arsenic species, followed by the transfer of a methyl group from a methyl donor. The generally held view of arsenic carcinogenesis in the past was that arsenite (As(III)) was the most probable cause of carcinogenesis and the methylation of arsenic species was a detoxification pathway (Tchounwou, 2003). Several authors agreed that methylation minimized the toxicity or carcinogenicity of arsenic (Kitchin, 2001). A recent alternative view of arsenic carcinogenesis, however, is that there may be several forms of arsenic that induce carcinogenesis and that arsenic methylation may be a toxification—not a detoxification—pathway (Tchounwou, 2003). (MMA(III)) has been found in urine of humans exposed to arsenic and this metabolite is known to inhibit enzymes and to cause cell toxicity and genotoxicity, and hence could be a potential cause of arsenic carcinogenesis (Tchounwou, 2003).

4.4.1 *Possible Mechanisms of Arsenic Carcinogenesis*

Kitchin discusses nine possible modes of action of arsenic carcinogenesis in his review: induced chromosomal abnormalities, oxidative stress, altered DNA repair, altered DNA methylation patterns, altered growth factors, enhanced cell proliferation, promotion/progression, suppression of *p*53, and gene amplification (Kitchin, 2001). Of these, three modes of action have shown positive results in both human and animal cell models: chromosomal abnormalities, oxidative stress, and altered growth factor (Tchounwou, 2003). The mode-of-action studies suggest that arsenic may be acting as a co-carcinogen, promoter, or a progressor of carcinogenesis (National Research Council, 2001).

4.4.2 *Animal Toxicity Studies*

To assess whether cytotoxicity and carcinogenicity of arsenic in animal models can be extrapolated to humans, it is necessary to have an understanding of the mode of action, metabolism, and toxicokinetics of different arsenic compounds in different animal species. Although a good animal model has yet to be found, rat and mouse models have been created for all human organs in which inorganic arsenic is known to cause cancer (skin, lung, urinary bladder, liver, and kidney) (Kitchin, 2001).

During the methylation pathway, inorganic arsenic undergoes a series of reductions and oxidative methylations in the human body to form the pentavalent

species (which are more easily excreted in the urine) to highly reactive and unstable trivalent metabolites. These trivalent species are monomethylarsinous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}). Exogenous arsenic in the form of MMA^{V} and DMA^{V} shows limited absorption and metabolism in humans and most animals (except rats) and are excreted mostly as parent compound (Cohen & Arnold, 2006). The rat is considered a poor model for arsenic metabolism because it is the only known animal model where a large proportion of the DMA is bound to red blood cells, leading to a decreased urinary excretion of this compound and increased accumulation in the body (Vasken Aposhian et al. 2004).

Animal models for DMA^{V}-induced promotion of carcinogenesis have been described for all five organs in which humans develop cancer post inorganic arsenic exposure; however, complete carcinogenesis by DMA^{V} has only been achieved in rat bladder and mouse lung (Kitchin, 2001). Arsenic as a promoter of carcinogenesis (but not complete carcinogenesis) has been achieved in mouse models for skin and lung cancer and in rat models for bladder, kidney, liver, and thyroid cancer (Kitchin, 2001). Rossman et al. (2001) showed that arsenite (trivalent arsenic) is a co-carcinogen for mouse skin in the presence of solar ultraviolet radiation, but arsenite alone did not cause tumors (Rossman et al. 2001). No carcinogenesis experiments have been reported with DMA^{V} exposure in hamsters, dogs, or monkeys (species that do not develop tumors after inorganic arsenic administration) (Kitchin, 2001). Among mammals that

methylate arsenic, humans excrete unusually high amounts of MMA, thus human tissues may be exposed to higher concentrations of pentavalent or trivalent MMA than are mice, rats, beagles, hamsters, or rabbits (Vahter, 1994).

4.4.3 *Dietary influence on Arsenic toxicity*

A dietary supplementation and arsenic toxicity study in mice (Singh et al. 2008) showed that treatment with arsenic trioxide and jaggery for a month reduced the frequencies of clastogenic endpoints compared to arsenic-treatment alone. Garlic as well as the fruit *Emblica officinalis,* also have been shown to significantly reduce the clastogenicity caused by arsenic administration in mice (Roychoudhury et al, 1996; Choudhury et al. 1997; Biswas et al. 1999). Poddar demonstrated that dietary iron supplementation also helps prevent arsenic toxicity by reducing its clastogenic effects (Poddar et al. 2000); selenium has similar antagonistic properties as iron-arsenic affinity and can ameliorate the arsenic-induced mutagenicity and clastogenicity in mice (Biswas et al. 1995; Rossman et al. 2004). A diet deficient in protein has been shown to decrease the arsenic methylation resulting in enhanced arsenic toxicity in rabbits (Vahter & Marafante, 1987). Other evidence indicates that oxidative stress is one of the major causes of arsenic toxicity. Studies in arsenic-exposed rats suggest that supplementation with vitamins E, C and A reduces the reactive species, helping reduce the toxic effects of arsenic (Wei et al. 2005; Ramanathan et al. 2005).

4.5 Effects of Arsenic on Human Health

Arsenic is known to be a human carcinogen. Several studies have suggested that arsenic toxicity in humans depends on the exposure dose, frequency and duration, age and gender as well as on individual susceptibility, which is influenced by genetic and nutritional factors (Chen & Lin, 1994). Long-term exposure to arsenic increases the risk of cancer. In workers exposed to arsenic via inhalation, the main carcinogenic effect is an increase in their risk of lung cancer (Enterline & Henderson, 1987); when exposure occurs via ingestion, the main carcinogenic outcome is increased risk of skin cancer. In addition, chronic exposure to arsenic can result in increased risk of several internal cancers, such as liver, kidney, lung, colon, and bladder (Tchounwou et al, 1999). Based on epidemiologic studies, chronic exposure to arsenic through contaminated drinking water also has non-carcinogenic effects on several organs and systems of the body including dermal, cardiovascular, reproductive, neurological, respiratory, hepatic, hematological, renal, and gastrointestinal (Tchounwou, 2003).

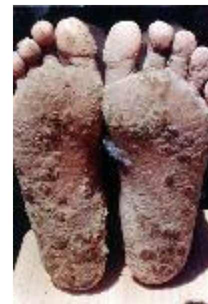


Figure 4.2: Arsenic-induced plantar skin lesion. Dhaka Community Hospital, Bangladesh Arsenic Foundation

Dermatologic effects: chronic arsenic exposure causes a characteristic pattern of noncarcinogenic dermal effects that starts with spots of hyperpigmentation and may progress to palmar and plantar hyperkeratosis (Mazumder, 1998).

These characteristic skin lesions induced by arsenic toxicity are used as an indicator of high exposure and are distinct from other clinical manifestations of arsenic toxicity (Tchounwou, 2003). A study by Mazumder et al (1998) found that males were more likely than females to exhibit both hyperpigmentation and palmar-plantar keratosis.

Cardiovascular Effects: Epidemiologic studies have shown that the cardiovascular system is especially sensitive to chronic exposure of arsenic contaminated drinking water. Some of the noticeable effects include hypertension and increased cardiovascular disease mortality. Rahman et al (1999) carried out a cross-sectional evaluation of blood pressure in 1,595 adults over the age of 30 who lived all their lives in rural Bangladesh and were exposed to high arsenic levels. The study found that increasing arsenic levels in drinking water were associated with increased incidence and severity of hypertension. There is also an increased risk of coronary heart disease, as individuals with blackfoot disease have increased mortality from ischemic heart disease (Chen, 1988). Blackfoot disease is a condition caused by peripheral atherosclerosis and thromboangiitis obliterans that results in gangrene and spontaneous amputation of the affected extremities (Tseng 1977).

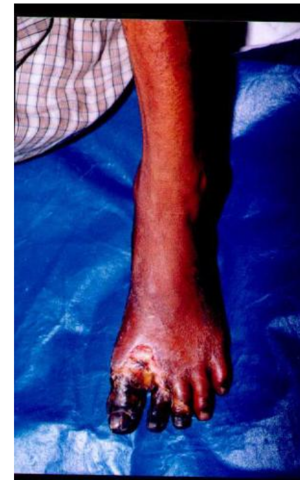


Figure 4.3: Arsenic-induced gangrene, (aka Blackfoot Disease).
Dhaka Community Hospital, Bangladesh

Hematologic Effects and Diabetes: Hernandez-Zavala et al studied the activities of enzymes of the heme biosynthesis pathway and their relationship with the profile of urinary protoporphyrin excretion in individuals exposed chronically to arsenic via drinking water in Mexico (Hernandez-Zavala et al, 1999). The more evident alterations observed in heme metabolism of highly exposed individuals included increases in porphobilinogen deaminase (PBG-D) and uroporphyrinogen decarboxylase (URO-D) activities in peripheral blood erythrocytes; increases in the urinary excretion of total porphyrins, mainly due to coproporphyrin III (COPRO-III) and uroporphyrin III (UROIII); and increases in the COPRO/URO and COPRO-III/COPROI ratios (Hernandez-Zavala et al, 1999). These results suggest that chronic arsenic exposure alters human heme metabolism. However, the biological mechanism responsible for these changes remains unknown.

4.5.1 *Effects of Arsenic on Female Reproductive Health*

Arsenic and its methylated metabolites have the ability to cross the placenta (Concha, 1998), and evidence from human studies suggests potential for adverse reproductive health effects. Nordstrom et al. studied offspring of female employees and nearby residents of a Swedish copper smelter where high levels of arsenic were documented (Nordstrom et al, 1978a; Nordstrom et al, 1978b; Nordstrom et al, 1979a; Nordstrom et al, 1979b). The offspring of women exposed to arsenic had lower birth weights than those of women who resided outside the smelter area, and the difference increased if the mothers worked in

the jobs with increased exposure (Nordstrom et al, 1979b). An incremental trend was noticed in the rates of spontaneous abortion with increasing exposure to arsenic (Nordstrom et al, 1978b; Nordstrom et al, 1979b). Congenital malformations were also seen more frequently if the pregnant mother was employed in high-exposure jobs during gestation (Nordstrom et al, 1979a).

Studies of populations affected by arsenic-contaminated drinking water have also found increased rates of spontaneous abortion and stillbirth (Borzsonyi et al. 1992; Castro, 1982). A study in Inner Mongolia of a population exposed to arsenic-contaminated drinking water showed that even low arsenic levels in the water (20-50 μg/L) were associated with increased systolic blood pressure in women six weeks post partum (Kwok et al. 2007). It was suggested that the cardiovascular challenge during pregnancy increased arsenic susceptibility (Vahter, 2009). There is also evidence that arsenic may cause anemia, especially during pregnancy, probably by destabilizing red blood cell membranes (Biswas D et al. 2008) and decreasing delta-aminolevulinic acid dehydratase activity (Kannan GM et al, 2001), a critical factor in heme synthesis.

4.5.2 *Gender Differences in Arsenic toxicity:*

In general, women have a higher fraction of DMA and a lower fraction of MMA in urine than men (Hopenhayn-Rich, 1996). This enhanced arsenic methylation in women has been shown to be limited to child-bearing age, since pre-pubertal and post-menopausal women showed methylation patterns similar to that of boys

and men, respectively (Lindberg, 2008). This difference may be related to involvement of female sex hormones in the arsenic methylation process, which may influence endogenous production of choline in women. After its oxidation to betaine, choline is the only source of methyl groups, besides folate, for the re-methylation of homocysteine to methionine (Vahter, 2009). Choline is synthesized in the body by SAM-dependent methylation of serine or recycled from lecithin (phosphatidylcholine), whose synthesis was recently shown to be up-regulated by estrogen; this may explain the efficiency of arsenic methylation in women in women of reproductive age (Vahter, 2009).

CHAPTER 5

BANGLADESH

Bangladesh is located in Southern Asia, bordering the Bay of Bengal between Burma and India. It comprises 144 thousand square kilometers, and is comparable in size to the U.S. state of Iowa (CIA, 2009). The estimated population as of 2009 is 156 million, making Bangladesh the eighth most dense country in the world (CIA, 2009), with an estimated 2,000 inhabitants per square mile (UN, 1999); its capital city is Dhaka.

The Bangladeshi population currently faces many public health challenges, one of the most important of which is arsenic poisoning. The contamination of groundwater by arsenic in Bangladesh is the largest poisoning of a population in known history, with an estimated 35 to 77 million persons at risk of drinking contaminated water (Smith et al, 2000). In the 1970s, the United Nation's Children's Fund (UNICEF) worked together with the Bangladeshi Department of Public Health to reduce morbidity and mortality from gastrointestinal diseases caused from drinking surface water contaminated with microbes and parasites. They installed tube wells that were 5-cm in diameter at a depth of less than 200 meters to provide the Bangladeshi population with "pure water" from

underground aquifers. At that time, arsenic contamination was not assumed to be a problem, and the water from these millions of wells was not tested for arsenic (Smith et al, 2000). The problem of arsenic contamination became evident 20 years later, when high arsenic levels were confirmed in 1993 upon testing the well water. It was found that 43 out of the 64 districts of Bangladesh have arsenic levels higher than 50µg/L, which is the maximum level permitted or considered to be "safe" in Bangladesh (Smith et al, 2000, Safiuddin, 2001); 59 out of the 64 districts have arsenic levels higher than 10 ug/L, which is the maximum level recommended by the WHO (Hossain M.F., 2006).

Table 4.1: Magnitude of arsenic poisoning in Bangladesh

Population of Bangladesh	125 million*
Total population in regions where some wells are known to be contaminated:	35-77 million
Maximum concentration of arsenic permitted in drinking water according to WHO recommendations:	10 µg/L
Maximum concentration allowed in Bangladesh:	50 µg/L
Number of tube wells sampled by the British Geological Survey (1998):	2,022
-Proportion of wells with arsenic concentrations >50 µg/L:	35%
-Proportion of wells with arsenic concentrations >300 µg/L:	8.4%

Smith A.H., Lingas E.O., & Rahman M. Contamination of drinking water by arsenic in Bangladesh: a public health emergency. Bulletin of the World Health Organization, 2000,**78**(9)
*Population of Bangladesh in year 2000

The Bangladeshi population exposed to these high levels of arsenic is at risk of developing arsenic poisoning, or arsenicosis. Arsenicosis causes painful cutaneous skin lesions, such as keratosis, hyper- or hypopigmentation, whose latency is typically 10 years, although the appearance of these skin lesions is thought to be dose-dependent (Guha Mazumder DN et al., 1998; Tondel M, Rahman M et al, 1999). Although the long-term effects of arsenic contamination

of drinking water appear to manifest slowly, which is why some of these negative health outcomes have only just begun to be observed, a great number of persons will develop these diseases in the future as a result of continuing exposure to arsenic. Currently there is an effort to determine whether nutritional status influences susceptibility to arsenicosis (Gamble et al. 2007; Milton et al. 2004; Li L. et al, 2008).

Another important issue in Bangladesh is the quality of life, as determined by morbidity and poverty. Close to 80% of the population lives in poverty, with a per capita income of $250 per year in the mid-1990s. Roughly 50% of the population survives on less than $1 per day, a percentage that is en route to being lowered to 30% in order to meet the Millennium Development Goals by 2015 (UN, 2005). The economy is based primarily on agriculture, and with the arsenic contamination issue, less land is able to be used, rendering the per hectare agricultural yield among the lowest in the world (Khuda Be & Helali J, 1991).

Malnutrition in Bangladesh is also a challenging issue, as it relates to maternal health, pregnancy outcome and childhood development. The cycle of maternal malnutrition, poverty, developmental delay and ill health all contribute greatly to a country's progress and economy. The current proportion of Bangladeshi mothers who are malnourished is estimated at 45% (WHO, 2005). The proportion of births attended by skilled birth professionals has risen from 5% to 12% in the past few years, but still remains unacceptably low (WHO, 2005). According to the WHO,

the maternal mortality ratio remains high at 570 deaths per 100,000 live births—a figure that is likely due in part to both malnutrition and arsenic exposure (WHO, 2005).

CHAPTER 6

PURPOSE OF THE STUDY

Iron is an essential nutrient, especially in women of reproductive age, as it can heavily affect reproductive health and pregnancy outcome. The female population of Bangladesh is likely at increased risk for iron deficiency, since there is a high prevalence of anemia. They are also at increased risk of arsenic toxicity in the drinking water. Arsenic alters heme metabolism and erythrocyte function by binding to hemoglobin, changing the shape of erythrocytes (Delnomdedieu et al. 1995; Lu et al, 2004; Winski & Carter, 1995) and lowering hemoglobin levels (Flora et al 2005; Kannan et al, 2001).

The effects of chronic arsenic toxicity on iron status remain largely unexplored. The main goal of this research project is to better understand the relationship between iron status, as measured by serum ferritin concentration, inflammation, and anemia in an arsenic-exposed cohort of reproductive-age women from Bangladesh. The results from this study may lead to a greater understanding of the determinants of iron status in Bangladeshi women of reproductive age and may aid in the creation of interventions aimed at improving the iron status of this population. The results from this study may also help to determine whether iron

status and inflammation status, as measured by hs-CRP, are affected by arsenic exposure.

6.1 Hypotheses:

- A large proportion of Bangladeshi women in this cohort will have depleted iron stores as determined by serum ferritin ≤12 micrograms/L.
- A large proportion of anemia, as determine by Hb<120 g/L, will be related to iron depletion.
- Although iron status may be compromised in women with arsenic-associated skin lesions (cases), inflammation will reduce our ability to detect iron depletion.
- Increased serum concentration of hs-CRP, a marker of inflammation, will be more common among cases than controls.

6.2 Specific Aims:

1. Measure serum concentrations of ferritin and identify factors associated with ferritin levels among women of reproductive age from the Pabna district of Bangladesh.
    - Possible factors that will be analyzed include education and height, weight, and body mass index (BMI).
2. Determine the percentage of women in this cohort with iron deficiency (ferritin ≤12 micrograms/L) and iron deficiency anemia (Hb <120 g/L and ferritin ≤12 micrograms/L).

3. Determine the percentages of cases and controls with elevated C-reactive protein (>3 mg/L)
4. Assess the relationship between iron deficiency, inflammation, anemia and risk of arsenic-associated skin lesions.
5. Determine whether C-reactive protein levels are useful in refining estimates of iron deficiency and iron deficiency anemia in women with and without arsenic-associated skin lesions.

CHAPTER 7

MATERIALS AND METHODS

7.1 Subjects

Researchers in the Department of Environmental Health at the Harvard School of Public Health (HSPH) conducted a study focused on arsenic exposure and skin lesions in Bangladesh (Breton et al. 2006). The study consisted of 1800 men and women (900 case-control pairs) recruited by Dhaka Community Hospital Trust primary-care clinics from 23 villages throughout the Pabna district of Bangladesh between 2001-2003. Up to 80% of controls from the initial HSPS study were selected from "low-exposure" arsenic (<50 mcg/L) areas and 20% of the subjects were from "high exposure" arsenic (≥ 50 mcg/L) areas in Pabna, based on the Bangladesh drinking water standard of 50 mcg/L. Participants with arsenic-induced skin lesions were only included in the study if a physician diagnosed any of the following: keratosis of the extremities; spotted melanosis; Bowen's disease; or squamous cell carcinoma. Controls consisted of men and women from that same area without any visible signs of arsenic-induced skin lesions.

The current research project included 147 women (75 cases, 72 controls) randomly chosen among female subjects 18 to 33 years of age who had serum archives of ≥ 1.3 mL.

7.2 Data Collection

At enrollment, anthropometric, behavioral and demographic data were obtained through a questionnaire administered by a community health worker.

7.2.1 Blood Sample Collection

Between 2001 and 2003, researchers from the Department of Environmental Health at the Harvard School of Public Health collected venous blood samples from all study subjects in Bangladesh. Hemoglobin was assayed from whole blood using *Sahli's* method. Serum was isolated by centrifugation, and aliquots were reserved, frozen and shipped on dry ice to HSPH where they were stored at -80◦ C. Frozen serum samples from 147 women were transported on dry ice to the Ronnenberg laboratory at the Department of Nutrition, University of Massachusetts Amherst, where they were stored at -80°C until biomarker assessment.

7.2.2 Laboratory Measurements

Ferritin concentration was determined using a commercially-available enzyme immunoassay kit from Ramco Laboratories Inc (Stafford, TX). The immunoassay results were read by an MRX Microplate Reader (Revelation) at 490 nm

wavelength with a correction filter set at 520 nm wavelength. In order to identify subjects who may have speciously elevated ferritin levels caused by infection or inflammation (and not sufficient iron status), high-sensitivity C-reactive protein (hs-CRP) levels were assessed using a commercially available immunoassay test kit from Biocheck, Inc (Foster City, CA). This assay has been validated by numerous researchers (Vikram NK, et al. 2004; Elkind et al 2006). Hs-CRP concentrations were read using the same microplate reader mentioned above at a wavelength of 450nm. Both ferritin and hs-CRP levels were plotted against a standard curve to determine biomarker concentrations, which were calculated using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California, USA).

Iron deficiency was defined as serum ferritin concentration equal to or below 12 micrograms/liter. Anemia was defined as hemoglobin levels under 120 grams/L, and iron-deficiency anemia was defined as hemoglobin under 120 grams/L and ferritin equal to or less than 12 micrograms/L. Iron deficiency in the presence of inflammation was defined as serum ferritin levels less than 50 micrograms/L if hs-CRP concentration exceeded 3mg/L.

All participants provided informed consent, and the study protocol was approved by Human Subjects Committees of the Harvard School of Public Health and the University of Massachusetts Amherst.

7.3 Statistical Analyses

The statistical analyses were carried out using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Descriptive data are presented as means +/- standard deviations, medians, ranges, and percentiles. The normality of our variables was assessed using both Kolmogorov-Smirnov & the Shapiro-Wilk tests of normality. Natural log transformation was applied to variables that were not normally distributed (serum ferritin, hs-CRP, water arsenic & toenail arsenic). Geometric means and their 95% confidence intervals are presented for logarithmically transformed data.

Physical and sociodemographic characteristics of the cases and controls were compared using the x^2 test for categorical data and the student t-test for continuous data. Simple linear regression and multivariable linear regression were performed to explore the relationship between serum ferritin and the same predictors mentioned above. Univariable and multivariable logistic regression models were used to explore the potential association between iron deficiency and the following potential predictors: age, height, weight, BMI, hs-CRP, toenail arsenic, water arsenic, education and marital status. Statistical significance was set at $p < 0.05$.

CHAPTER 8

RESULTS

Our study included 147 women from the Pabna district in Bangladesh. The demographic characteristics of the population by case-control status are presented in table 8.1. The women in this study ranged from 18-33 years in age. The mean height was 152 cm (4'9") and weight was 102 pounds, resulting in an average BMI of 20.2 kg/m^2. Almost a third (30%) of the women were classified as underweight with a BMI<18.5 kg/m^2, and most of the remaining had a BMI between 18.5 kg/m^2-25 kg/m^2. The majority of the participants (73%) were married. Thirty-six percent of the study population had no formal education. This analysis cohort includes cases (N=75) and controls (N=72) based on the presence of arsenic-associated skin lesions (table 8.1).

Because serum ferritin was not normally distributed according to the Kolmogorov-Smornov (p=0.000) and Shapiro-Wilk (p=0.00) tests of normality, data were transformed to their natural logarithm (ln). After ln transformation, the Kolmogorov-Smornov (p=0.2) showed a deviation from normality that was not significant, whereas the Shapiro-Wilk (p=0.03) test showed a significant deviation from normality in the log-transformed data.

Serum ferritin concentration ranged from 0.8 μg/L to 224.5 μg/L, with a geometric mean of 28.0 μg/L. Overall, 18.4% of the women had iron depletion (serum ferritin ≤12 μg/L). About a fifth of our sample had anemia (17.8%) as determined by hemoglobin <120 g/L (World Health Organization Standard).
The main serological difference between women with arsenic-associated skin lesions (cases) and those without (controls) was the mean serum hemoglobin (124 g/L vs 129 g/L, respectively; p=0.02) as seen in table 8.1. Cases were also more likely to have inflammation (p= 0.04) and have a higher toenail arsenic concentration (p= 0.00).

The proportion of women who had iron depletion was lower in women ages 28-33 compared to those ages 18-22 years old (p=0.00). Married women were also less likely than unmarried women to have iron depletion (12.7% vs 35%, respectively; p=0.01). The prevalence of inflammation was 21.4%, as determined by high-sensitivity c-reactive protein levels over 3 mg/L (which was also log-transformed). Women with normal serum ferritin levels were also more likely to have inflammation compared to those who were iron deplete; however, this was not statistically significant. Over half of the study population's main water source contained arsenic levels over the WHO health standard of 10 μg/L, and about a third (32.9%) of the participants' main water source contained arsenic levels over the safety standard for Bangladesh (>50μg/L). Arsenic data were also skewed and log transformed for our analysis (Table 1).

TABLE 8.1: Characteristics of Controls and Cases in our study

Continuous Variables	Mean ± SD (N=147)	Controls (N=72) Mean ± SD	Cases (N=75) Mean ± SD	P-value (T-test)
Age (years)	25.5 ± 5.0	25.7 ± 5.3	25.4 ± 4.74	0.66
Height (cm)	152.0 ± 5.7	151.9 ± 6.0	152.1 ± 5.7	0.83
Weight (kg)	47.2 ± 10.6	48.6 ± 13.2	46.0 ± 7.1	0.13
BMI (kg/m^2)[c]	20.2 ± 3	20.5 ± 3.0	19.9 ± 2.9	0.22
Ferritin (µg/L)*	28.2 (24.2, 32.9)	27.8 (21.7, 33.0)	29.8 (23.9, 37.3)	0.48
Hemoglobin (g/L)[c]	126.4 ± 12.8	128.9 ± 10.1	123.9 ± 14.5	0.02
hs-CRP (mg/L)*[c]	0.7 (0.5, 1.0)	0.44 (0.23, 0.85)	1.0 (0.65, 1.7)	0.04
Water As (µg/L)*[c]	15.3 (10.2, 23.0)	12.3 (7.6, 19.8)	18.9 (9.7, 36.9)	0.33
Toenail As (µg/g)*[c]	2.5 (2.1, 3.0)	1.8 (1.5, 2.1)	3.5 (2.6, 4.8)	0.00
Categorical Variables	**Total N (%)**	**Controls N (%)**	**Cases N (%)**	**P-value**
Age				
18-22	50 (34)	25 (50)	25 (50)	0.63
23-27	42 (28.6)	18 (42.9)	24 (57.1)	
28-33	55 (37.4)	29 (52.7)	26 (47.3)	
BMI				
<18.5 kg/m^2	44 (30.1)	15 (34.1)	29 (65.9)	0.07
18.5-24.9 kg/m^2	91 (62.3)	50 (54.9)	41 (45.)	
>25 kg/m^2	11 (7.5)	6 (54.5)	5 (45.5)	
Iron status				
Normal ferritin	120 (81.6)	58 (48.3)	62 (51.7)	0.83
Iron deplete (ferritin ≤12ug/L)	27 (18.4)	14 (51.9)	13 (48.1)	
Water Arsenic				
≤10 ug/L	60 (42.9)	29 (48.3)	31 (51.7)	0.01
10-50 ug/L	34 (24.3)	24 (70.6)	10 (29.4)	
>50 ug/L	46 (32.9)	17 (37.0)	29 (63.0)	
hs-CRP (mg/L)				
Normal (<3 mg/L)	114 (78.6)	58 (50.9)	56 (49.1)	0.42
High (≥3 mg/L)	31 (21.4)	13 (41.9)	18 (58.1)	
Hemoglobin (g/L)				
Normal (Hb≥120 g/L)	120 (82.2)	64 (53.3)	56 (46.7)	0.02
Anemia (Hb<120g/L)	26 (17.8)	7 (26.9)	19 (73.1)	
Marital Status				
Single/Unmarried	37 (25.2	18 (45.9)	19 (54.1)	0.83
Ever Married	110 (74.8)	55 (50.0)	55 (50.0)	
Education				
No formal education	53 (36.1)	23 (43.4)	30 (56.6)	0.60
1^{ary} or 2^{ary} Education	69 (46.9)	36 (52.2)	33 (47.8)	
Higher secondary Educ.	25 (17.0)	13 (52.0)	12 (48.0)	

*presenting geometric means (exponentiated natural log mean values, and 95% CI)

[c] missing one or more values: BMI: N=146 (controls:71), CRP:N=145 (controls:71; cases:74), Hb: N=146 (controls:71)

TABLE 8.2: Characteristics of Bangladeshi Women in our Study Sample
(categorized by iron status)

Continuous Variables	Mean ± SD (N=147)	Ferritin >12ug/L (N=120) Mean ± SD	Ferritin≤12ug/L (N=27) Mean ± SD	P-value (T-test)
Age (years)	25.5 ± 5.0	26 ± 4.8	22 ± 4.8	0.001
Height (cm)	152.0 ± 5.7	151.9 ± 5.8	152.2 ± 5.3	0.85
Weight (kg)	47.2 ± 10.6	46.4 ± 7.5	46.8 ± 6.2	0.80
BMI (kg/m^2)[c]	20.2 ± 3	20.1 ± 3.06	20.2 ± 2.5	0.96
Ferritin (ug/L)*	28.2 (24.2, 32.9)	38.7 (34.2, 43.7)	7.1 (5.5, 9.0)	0.000
Hemoglobin (g/L)[c]	126.4 ± 12.8	126.5 ± 11.9	126.5 ±16.5	0.95
hs-CRP (mg/L)*[c]	0.7 (0.5, 1.0)	0.9 0.6,1.3)	0.25 (0.1,0.8)	0.02
Water As (ug/L)*[c]	15.3 (10.2, 23.0)	18.8 (12.0, 29.5)	6.2 (2.4, 15.9)	0.03
Toenail As (ug/g)* [c]	2.5 (2.1, 3.0)	2.9 (2.3, 3.6)	1.4 (1.1, 1.8)	0.00

Categorical Variables	Total N (%)	Ferritin >12ug/L N (%)	Ferritin ≤12ug/L N (%)	P-value
Age				
18-22	50 (34)	34 (68)	16 (32)	0.00
23-27	42 (28.6)	35 (83.3)	7 (16.7)	
28-33	55 (37.4)	51 (92.7)	4 (7.3)	
BMI				
<18.5 kg/m^2	44 (30.1)	39 (88.6)	5 (11.4)	0.36
18.5-24.9 kg/m^2	91 (62.3)	71 (78.0)	20 (22.0)	
>25 kg/m^2	11 (7.6)	9 (81.8)	2 (18.2)	
Case Status				
Control	72 (49)	58 (80.6)	14 (19.4)	0.83
Case	75 (51)	62 (82.7)	13 (17.3)	
Water Arsenic				
≤10 ug/L	60 (42.9)	45 (75.0)	15 (25)	0.18
10-50 ug/L	34 (24.3)	28 (82.4)	6 (17.7)	
>50 ug/L	46 (32.9)	41 (89.1)	5 (10.9)	
hs-CRP (mg/L)				
Normal (<3 mg/L)	114 (78.6)	89 (78.1)	25 (21.9)	0.07
High (≥3 mg/L)	31 (21.4)	29 (93.5)	2 (6.5)	
Hemoglobin (g/L)				
Normal (Hb≥120 g/L)	120 (82.2)	100 (83.3)	20 (16.7)	0.27
Anemia (Hb<120g/L)	26 (17.8)	19 (73.1)	7 (26.9)	
Marital Status				
Unmarried	37 (25.2)	24 (64.9)	13 (35.1)	0.01
Ever married	110(74.8)	96 (87.3)	14 (12.7)	
Education				
No formal education	53 (36.1)	46 (86.8)	7 (13.2)	0.28
1^{ary} or 2^{ary} Education	69 (46.9)	56 (81.2)	13 (18.8)	
Higher secondary Educ.	25 (17.0)	18 (72.0)	7 (28)	

*presenting geometric means (exponentiated natural log mean values, and 95% CI)
[c] missing one or more values (BMI: N=146, CRP:N=145, Hb: N=146, Water As: N=139, toenail As: N=139)

A summary of the dietary patterns of this cohort is shown in Table 8.3. Low quality of the dietary data impeded us from carrying out further analysis in this dataset.

Table 8.3 Dietary factors associated with Iron deficiency (serum ferritin ≤12 ug/L)

Consumption frequency of each food:	Total (N=147)	Normal Iron status (ferritin >12ug/L) (%)	Iron Depletion (ferritin ≤12ug/L) (%)	p-value
Fish:				
None to < 3/monthly	4	2 (50%)	2 (50%)	0.15
1-6 times weekly	128	104 (81.3%)	24 (18.8%)	
Once or more per day	15	14 (93.3%)	1 (6.7%)	
Fowl:				
None/Almost none	26	19 (73.1%)	7 (26.9%)	0.43
1-3/month	105	88 (83.8%)	17 (16.2%)	
1-6 weekly	16	13 (81.3%)	3 (18.8%)	
Beef :				
None/Almost none	31	24 (77.4%)	7 (22.6%)	0.62
1-3/month	92	77 (83.7%)	15 (16.3%)	
1-6 weekly	24	19 (79.2%)	5 (20.8%)	
Egg :				
None/Almost none	19	16 (84.2%)	3 (19.3%)	0.63
1-3/month	48	37 (77.1%)	11 (22.9%)	
1-6 weekly or 1-2 daily	80	67 (83.8%)	13 (16.2%)	
Bean :				
None/Almost none	135	109 (80.7%)	26 (19.3%)	0.79
1-3/month to 1-6 weekly	10	9 (90.0%)	1 (10.0%)	
1 or more times daily	2	2 (100%)	0 (0%)	
Vegetables:				
None to <3/month	8	4 (50.0%)	4 (50.0%)	
1-6 weekly	102	85 (83.3%)	17 (16.7%)	0.09
One or more daily	37	31 (83.8%)	6 (16.2%)	
Milk :				
None/Almost none	30	19 (63.3%)	11 (36.7%)	0.02
1-3 times per month	40	34 (85.0%)	6 (15.0%)	
1-6/week to1-2/day	77	67 (87.0%)	10 (13.0%)	
Rice :				
None/Almost none	35	34 (97.1%)	1 (2.9%)	0.01
1-3/month to 1-2/day	27	22 (81.5%)	5 (18.5%)	
3+ per day	85	64 (75.3%)	21 (24.7%)	
Bread :				
None/Almost none	82	61 (74.4%)	21 (25.6%)	0.02
1-3/month to 1-2/day	36	31 (86.1%)	5 (16.1%)	
3 or more/day	29	28 (96.6%)	1 (3.4%)	

We conducted univariable linear regression to evaluate the associations between potential covariates and (natural log) serum ferritin (table 8.4). Age, inflammation (hs-CRP), married status, toenail As and As in water were each significantly and positively associated with serum ferritin. In our multivariable linear regression, only hs-CRP (p=0.00), toenail arsenic levels (p=0.01) and marital status (p=0.001) were significant predictors of serum ferritin after adjustment for other variables.

Table 8.4: Linear regression: predictors of serum ferritin
(Y=natural log of serum ferritin)

	Univariable Model		**Multivariable Model 1**		**Multivariable Model 2**	
	Coefficient	**p-value**	**Coefficient**	**p-value**	**Coefficient**	**p-value**
Age (yrs)	0.04	0.004[a]	0.02	0.48		
Height (cm)	-0.02	0.14[a]	-0.02	0.21		
Hs-CRP[c]	0.12	0.000[a]	0.11	0.000[b]	0.11	0.000
Toenail As[c] (µg As/g toenail)	0.22	0.002[a]	0.15	0.06[b]	0.16	0.017
Arsenic in water						
≤10ug/L	Ref		Ref			
10-50ug/L	0.22	0.27	0.13	0.52		
>50ug/L	0.45	0.01[a]	0.04	0.84		
Education						
No formal Educ.	Ref		Ref			
1^{ary} or 2^{ary} Educ.	-0.25	0.13	-0.05	0.79		
Higher 2^{ary} Educ.	-0.49	0.03[a]	-0.12	0.68		
Marital Status						
Not Married	Ref		Ref		Ref	
Ever married	0.71	0.00[a]	0.40	0.08[b]	0.55	0.001

a- Included in 1st multivariable linear regression model (p-value <0.25)
b- Included in 2nd multivariable linear regression model (p-value <0.10)
c- used transformed value (natural log transformation)

We used univariable logistic regression to identify factors crudely associated with iron depletion, defined as serum ferritin ≤12μg/L (table 8.5). From this analysis, we observed a strong association between increasing age and decreasing risk of iron depletion. Women in the oldest tertile were 81% less likely than women in the youngest tertile to have iron depletion (p=0.006). Marital status was also related to iron depletion (Table 8.6), as women who were married were 73% less likely to have iron depletion (p=0.003, 95%CI: 0.11, 0.65). Women in the highest tertile of toenail arsenic concentration were 84% less likely to have iron depletion (p=0.007) compared to those in the lowest tertile. Those whose water originated from wells containing the highest amounts of arsenic were 75% less likely to have iron depletion (p=0.015) compared to those whose water arsenic content was in the lowest tertile. No other significant crude associations were observed in this analysis.

From our multivariable logistic regression analysis in table 8.7, the only significant predictors of iron depletion were age, BMI, toenail arsenic, and inflammation after controlling for other variables. Women between 29-33yrs old were 84% less likely to have iron depletion compared to women ages 18-22 (OR=0.16; 95%CI=0.04, 0.56). Women with inflammation were 80% less likely to have low ferritin compared to those without inflammation (OR=0.20, 95%CI=0.04, 0.96). Women who were normal or over-weight (BMI>18.5kg/m^2) were nearly four times more likely to have iron depletion (OR=3.72, 95%CI=1.17, 11.87) than were women with a lower BMI. In addition, r every 1μg As increase per gram of toenail was associated with a 45% lower risk of ID (OR=0.55, 95%CI=0.33, 0.94).

Table 8.5: Crude Associations with events of Iron Depletion (ferritin ≤12 µg/L), by tertiles

	N	# events (%)	OR	95% CI	p-value
Age (years)					
18-22	50	16 (32)	Ref		
23-28	48	7 (14.6)	0.36	0.13, 0.98	0.046
29-33	49	4 (8.2)	0.19	0.06, 0.62	0.006
Height (cm)					
<150	56	10 (17.9)	Ref		
151-154	49	11(22.5)	1.33	0.51, 3.47	0.56
154-166	42	6 (14.3)	0.77	0.26, 2.31	0.64
Weight (lb)					
<97	50	8 (16)	Ref		
97.1-106	48	8 (16.7)	1.08	0.37, 3.14	0.895
106.1-156	48	11 (22.9)	1.60	0.58, 4.39	0.364
BMI (kg/m^2)					
<18.9	48	7 (14.6)	Ref		
18.9-20.6	49	9 (18.4)	1.35	0.46, 3.97	0.585
>20.6	49	11 (22.5)	1.74	0.61, 4.94	0.300
CRP (mg/L)					
<0.5	49	12 (24.5)	Ref		
0.5-1.8	48	10 (20.8)	0.79	0.30, 2.05	0.63
1.8-51	48	5 (10.4)	0.34	0.11, 1.06	0.06
Hb (g/L)					
<120	53	8 (5.1)	Ref		
121-130	50	10 (20)	1.44	0.52, 3.99	0.486
130-170	43	9 (20.1)	1.52	0.53, 4.35	0.433
Toenail As (µg/g)					
0.1-1.3	47	14 (29.8)	Ref		
1.31-3.49	48	10 (20.8)	0.62	0.24, 1.58	0.32
3.5-53.75	47	3 (6.4)	0.16	0.04, 0.61	0.007
As in water (µg/L)					
11-46	46	15 (32.6)	Ref		
47-94	48	6 (12.5)	0.30	0.10, 0.85	0.023
95-140	48	5 (10.4)	0.25	0.08, 0.77	0.015

Table 8.6.Crude associations between socio-demographic factors and ID

	N	# events (%)	OR	95% CI	p-value
Education					
No formal Education	53	7 (13.2)	Ref		
$1^{ary}/2^{ary}$ Education	69	13 (18.8)	1.53	0.56, 4.14	0.41
Higher 2^{ary} education	25	7 (28)	2.56	0.79, 8.3	0.12
Marital Status					
Not married	37	13 (35.1)	Ref		
Ever Married	110	14 (12.7)	0.27	0.11, 0.65	0.003

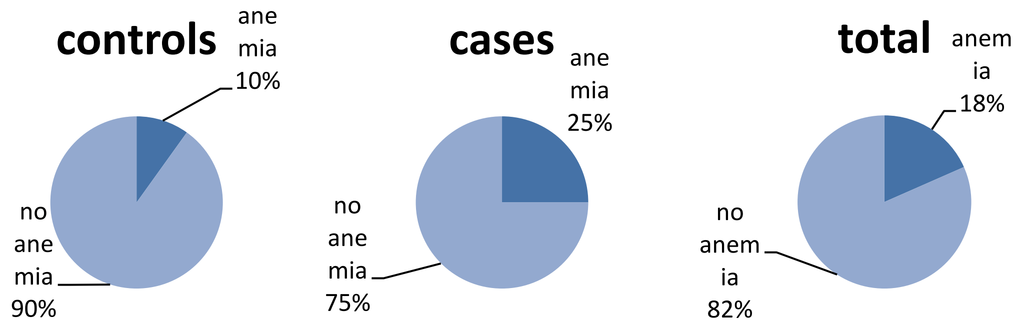
Table 8.7: logistic regression: predictors of ID (Y=Iron Depletion)

	Model 1 (univariable)		Model 2 (includes p <0.25)		Model 3 (includes p<0.10)	
	OR	**p-value**	**OR**	**p-value**	**OR**	**p-value (95% CI)**
<u>Age</u>						
18-22	Ref		Ref		Ref	
23-28	0.36	0.046[a]	0.38	0.13	0.40	0.11 (0.13, 1.23)
29-33	0.19	0.006[a]	0.16	0.02[b]	0.16	0.01(0.04, 0.56)
Height (cm)	1.007	0.85				
Weight (kg)	0.99	0.80				
<u>BMI (kg/m^2)</u>						
Underweight	Ref		Ref		Ref	
Normal weight/above	2.09	0.17[a]	5.02	0.01[b]	3.72	0.03 (1.17, 11.87)
Toenail As levels (µg/g)[c]	0.48	0.005[a]	0.55	0.04[b]	0.55	0.03 (0.33, 0.94)
<u>Arsenic in water</u>						
≤10µg/L	Ref		Ref			
10-50µg/L	0.68	0.48	0.60	0.42		
>50µg/L	0.39	0.09[a]	0.66	0.53		
<u>Hs-CRP</u>						
No inflammation	Ref		Ref		Ref	
Inflammation	0.25	0.07[a]	0.20	0.06[b]	0.20	0.05 (0.04, 0.96)
<u>Education</u>						
No formal Education	0.7	0.41	Ref			
1^{ary} or 2^{ary} Educ	1.00		0.60	0.45		
Higher 2^{ary} Educ	1.7	0.34	0.33	0.25		
<u>Marital Status</u>						
Not Married	1.00		Ref			
Married	0.31	0.009[a]	0.35	0.11		

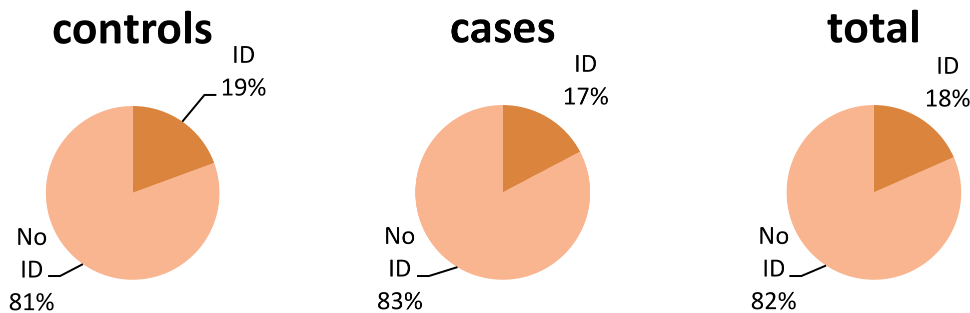
a- Included in 1st multivariable logistic regression model (p-value <0.25)
b- Included in 2nd multivariable logistic regression model (p-value <0.10)
c- used transformed value (natural log transformation)

Of the 147 women in the cohort, 26 had anemia (18%), as determined by the World Health Organization cutoff of hemoglobin levels less than 120 g/L (graph 8.1). The prevalence of iron depletion, as determined by serum ferritin <12 µg/L, was 18% (N=27) as observed in graph 8.2.

Graph 8.1: Prevalence of anemia in our study. Anemia, as defined by hemoglobin levels <120 g/L among this cohort is 18%. The difference in prevalence of anemia among controls and cases is statistically significant, with controls having a prevalence of anemia of 10% (N=7) vs 25% (N=19) in cases (p= 0.02)

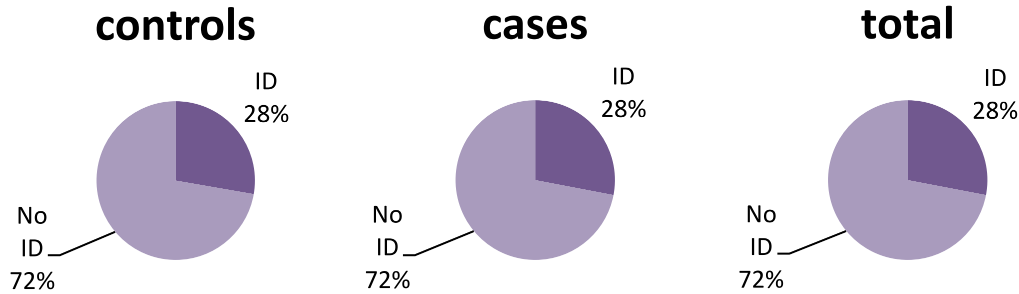


Graph 8.2: Prevalence of iron deficiency in our study. ID, as defined by serum ferritin ≤12 ug/L, is 18% (N=27). The distribution of iron deficiency between controls and cases is similar, 19% (N=14) vs 17% (N=13), respectively.

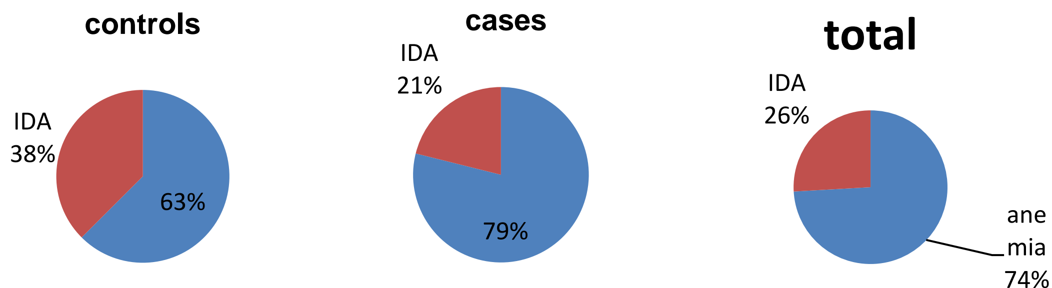


Cases have higher levels of inflammation, determined by serum c-reactive protein (p=0.04). The higher prevalence of inflammation in this group of women with arsenic-associated skin lesions could possibly mask iron depletion. In order to determine whether inflammation could be masking iron depletion, we extended the cutoff for 'adequate' iron stores to 50µg ferritin/L in the presence of inflammation, defined as hs-CRP over 3 mg/L.

Graph 8.3: Prevalence of iron deficiency with inflammation. ID increases once inflammation is accounted for. The new cutoff for iron depletion (<50 ug/L if hs-crp >3mg/L) increases the proportion of women with iron depletion in both cases and controls. Controls increase from 19% (N=14) to 28% (N=20), and cases from 17% (N=13) to 28% (N=21). Total ID prevalence increases from 18% (N=27) to 28% (N=41).



Graph 8.4: Prevalence of iron deficiency anemia. Among the women with anemia (N=26), the prevalence of anemia concurrent with iron depletion (Iron Deficiency Anemia, or IDA) varies by case-control status. Of the 7 controls with anemia, 38% (N=3) have IDA; of the 19 cases with anemia, 21% (N=4) have IDA. The total prevalence of IDA among the women with anemia is 26% (N=7). Total prevalence of IDA in this cohort is 4.8% (N=7)



The proportion of women who now had iron depletion increased from 18% (N=27) to 28% (N= 41) as observed in graph 8.3. Among the 26 women with anemia (Graph 8.4), only 26% were also iron deficient (iron deficiency anemia). The prevalence of IDA did not change after the cut-off form iron deficiency was extended in the presence of inflammation (table 8.8). The only predictor of anemia was case status (table 8.9; p=0.02).

Table 8.8: Proportion of women with Iron depletion using two different serum ferritin cutoff points to account for the presence of inflammation.

	Controls (N=72)	**Cases (N=75)**	**Total (N=147)**
Iron Depletion (ID) cutoff:			
Ferritin <12 ug/L	14 (51.9%)	13 (48.1%)	27 (18.4%)
Ferritin <50 ug/L (+ hs-CRP >3mg/)	20 (48.8%)	21 (51.2%)	41 (28.3%)[b]
Anemia (Hb<120g/L only)	7 (26.9%)[a]	19 (70.4%)	26 (17.7%)
The following percentages are out of the total number of women with Anemia, as determined by Hemoglobin less than 120 g/L (N=26)			
IDA (Hb<120g/L+ Iron depletion)			
Ferritin <12 ug/L	3 (42.9%)	4 (57.1%)	7 (26.9%)
Ferritin <50 ug/L (+ hs-CRP >3mg/)	3 (42.9%)	4 (57.1%)	7 (26.9%)

[a]Controls included =71

Table 8.9: Logistic regression: predictors of Anemia (Y=Hb<120g/L)

	Model 1 (univariable)		**Model 2** (includes p <0.25)		**Model 3** (includes p<0.10)	
	OR	**p-value**	**OR**	**p-value**	**OR**	**p-value (95% CI)**
Age						
18-22	Ref		Ref			
23-28	1.92	0.23*	2.23	0.17		
29-33	1.23	0.71	1.69	0.39		
Height (cm)	0.97	0.49				
Weight (kg)	1.01	0.37				
BMI (kg/m^2)						
Underweight	Ref					
Normal weight/above	1.19	0.72				
Case Status						
Control	Ref		Ref		Ref	
Case	3.1	0.02*	3.23	0.02[b]	3.1	0.02 (1.21, 7.92)
Toenail As (µg/g) [c]	1.02	0.94				
Arsenic in water						
≤10µg/L	Ref					
10-50µg/L	0.71	0.56				
>50µg/L	0.60	0.35				
Iron status:						
Normal ferritin (>12ug/L)	Ref		Ref			
Low ferritin (<12ug/L)	1.8	0.23[a]	2.4	0.11		
Serum ferritin (ug/L)[C]	0.84	0.45				
Hs-CRP						
No inflammation	Ref					
Inflammation	0.78	0.65				
Education						
No formal Education	Ref					
1[ary] or 2[ary] Educ	0.59	0.27				
Higher 2[ary] Educ	0.65	0.50				
Marital Status						
Not Married	Ref					
Married	1.53	0.43				

[c]-used transformed value (natural log transformation)

Table 8.10: Logistic regression: predictors of Arsenic-associated skin lesions (Y=case/control status)

	Model 1 (univariable)		**Model 2** (includes p <0.25)		**Model 3** (includes p<0.10)	
	OR	**p-value**	**OR**	**p-value**	**OR**	**p-value (95% CI)**
Age						
18-22	Ref					
23-28	1.33	0.49				
29-33	0.90	0.78				
Height (cm)	1.006	0.83				
Weight (kg)	0.97	0.16[a]	1.01	0.86		
BMI (kg/m^2)						
Underweight	Ref		Ref		Ref	
Normal weight/above	0.397	0.015[a]	0.34	0.05[b]	0.36	0.02 (0.15, 0.86)
Case Status						
Control						
Case						
Toenail As (µg/g)[c]	1.86	0.001[a]	2.29	0.001[b]	3.0	0.001 (1.43, 3.70)
Arsenic in water						
≤10µg/L	Ref		Ref		Ref	
10-50µg/L	0.39	0.04[a]	0.26	0.012[b]	0.25	0.01 (0.09, 0.73)
>50µg/L	1.60	0.24[a]	0.97	0.95	0.95	0.92 (0.35, 2.61)
Anemia						
Hb>120g/L	Ref		Ref		Ref	
Hb<120g/L	3.10	0.018[a]	7.12	0.002[b]	7.13	0.004 (2.09,24.3)
Ferritin status						
Normal (>12ug/L)	Ref					
Low (<12ug/L)	0.87	0.74				
Education						
No formal Education	Ref					
1[ary] or 2[ary] Educ	0.70	0.34				
Higher 2[ary] Educ	0.71	0.48				
Marital Status						
Not Married	Ref					
Married	0.85	0.67				

[c]-used transformed value (natural log transformation)

Higher toenail As levels (p=0.001) and anemia (p=0.00) were the two strongest predictors of arsenic-associated skin lesions after adjusting for other variables (table 8.10).

CHAPTER 9

DISCUSSION

Our study focused on young women of reproductive age from the District of Pabna in Bangladesh. The prevalence of anemia in Bangladesh has been estimated by others to surpass 70% of the population, affecting approximately 45% of the female, non-pregnant population (Ahmed, 2000). The prevalence of anemia, as determined by the WHO standard of less than 120 g/L, was much lower in our study group (17.8%). This could be in part due to the method of hemoglobin assessment used in the field, *Sahli's* method. This colorimetric method of hemoglobin assessment has a potential for error rate due to subjective bias in visual comparison to determine concentration (Balasubramaniam et al, 1992). *Sahli's* method has been found to have a high sensitivity (92.3% and 98%), but a low specificity (39% and 66%) (Barduagniha et al, 2003; Anand et al, 2009). In our study population, there were 27 subjects whose hemoglobin concentration was exactly 120g/L, leading to our classifying them as not anemic. It remains unclear what proportion of these subjects were actually anemic, but had their hemoglobin levels rounded up to 120g/L, but had we included them in our anemic pool, the estimated prevalence of anemia would have increased to 36%. However, more recent studies have declared *Sahli's* method to be in better

agreement [than other field hemoglobin tests] with autoanalyzer methods, which remain the gold standard for hemoglobin assessment (Anand et al, 2009).

The WHO estimates that approximately half of the cases of anemia are due to iron deficiency. In our study, approximately one fourth (26.9%) of the subjects with anemia (N=26) had iron deficiency anemia (N=7), as defined by concurrent anemia (Hb<120g/L) and iron depletion (ferritin≤12μg/L). It has been assumed that half of the cases of anemia in developing countries are due to iron depletion, but we found this may not be the case in Bangladesh. Ahmed found that severe anemia is less frequent in Bangladesh (2-3%); we did not find any subjects with hemoglobin levels less than 90 g/L, so this was in agreement with what is currently known (Ahmed, 2000). However, according to the data on the etiology of anemia that Ahmed analyzed, iron deficiency seemed to be a substantial cause of anemia in the Bangladeshi population (Ahmed, 2000). This did not appear to be the case in our study population. Due to the nature of our study location, we adjusted the cut-off for defining iron depletion upward to account for the influence of inflammation or infection on ferritin, given that ferritin is an acute-phase protein and infection may be more prevalent in Bangladesh. Bangladesh has high prevalence of communicable diseases, such as malaria (26 million people are at risk), tuberculosis (300,000 new cases per year), leprosy (Dhaka still has some cases of leprosy), and filiariasis, which is a problem in the northern districts of Bangladesh (WHO, 2010). Previous studies have extended the normal 12-15 μg ferritin/L cutoff to 30-50μg/L to account for infection or inflammation

(Beard et al, 2006). We categorized those women who had ferritin levels <50μg/L in the presence of inflammation (hs-CRP >3mg/L) as iron depleted. This resulted in an increased sensitivity for diagnosing iron depletion, at the cost of possible decreased specificity. The new cut-off increased our number of iron depleted women from 27 to 41 (18.4% to 28.3%), but the prevalence of IDA remained the same.

The strongest predictors of serum ferritin levels were serum c-reactive protein (p=0.000), marital status (p=0.001) and toenail arsenic levels (p=0.02). Ferritin, being an acute-phase reactant, is elevated in states of infection, inflammation, neoplasia, hepatic dysfunction and alcohol consumption (Crichton, 2006). Therefore the observation of an association between the marker of inflammation, c-reactive protein, and serum ferritin was not surprising. The average age for married women in our study was 27 years, whereas unmarried women were 20.9 years old. We observed a significant decrease in risk of iron deficiency as women grew older; women who were in the oldest group (ages 29-33) were 84% less likely to have iron depletion compared to the youngest group (ages 18-22) (p=0.01). Hence, the association with marital status is most likely due to the difference in age.

The association between toenail arsenic concentration and serum ferritin levels was unexpected. Women who had normal iron stores (serum ferritin>12μg/L) had twice the toenail arsenic concentration of those who were iron depleted (2.9 μg

arsenic/gram toenail compared to 1.4 µg arsenic/gram toenail) (p=0.00). This observation could be due to the presence of high levels of iron in the same tubewell water where arsenic is prevalent. The national hydro-chemical survey of groundwater conducted by the British Geological Survey (BGS) and the Department of Public Health Engineering (DPHE) in Bangladesh have shown that a large proportion of wells exceed permissible limits for both iron and arsenic (BGS, DPHE, 2001). The permissible limit of iron for drinking water is 300µg/L to 1.0mg/L, but in Bangladesh, Hossain reported that 41% and 25% of the tubewells studied exceeded iron concentrations of 1.0mg/L and 5.0 mg/L, respectively (Hossain et al, 1997). In the district of Dhaka, the correlation between arsenic and iron concentrations was significant with a correlation coefficient of 0.47 p =0.0001 (Tonmoy et al, 2009). We found that the mean arsenic content (µg/L) in the tubewell water of women with normal ferritin levels was three times higher than the tubewell water of women who were iron deplete (18.8 µg arsenic /L compared to 6.2 µg arsenic/L, p=0.03). However, arsenic water concentration was not a predictor of iron status. A possible explanation for the absence of association could be the lack of water consumption or usage data. The food frequency questionnaire used to collect the dietary data did not include water intake.

We did not observe any significant associations between dietary factors and iron status or arsenic toxicity. However, there was a slight trend toward decreased iron deficiency among women who consumed more fish, fowl and beef compared

to those who consumed less of these animal foods. According to Kile et al, the dominant source of arsenic exposure in women living in Pabna is the water consumed (Kile et al, 2007). They also observed that as arsenic concentrations in the drinking water decrease, the relative contribution of dietary arsenic sources are more relevant to ingested doses. Therefore, the combined intake from both diet and drinking water can result in arsenic consumption that exceeds the WHO's provisional tolerable daily intake of 2.1 pg As/kg-day, despite using tube well that contains less than 50μg As/L (Kile et al, 2007).

In our study population, we also had 75 subjects with As-associated skin lesions (cases) and 71 without skin lesions (controls). The women with As-associated skin lesions had a slightly higher mean serum ferritin concentration, but this was not significant (27.8 μg/L compared to 29.8 μg/L, p=0.48). However, the mean c-reactive protein concentration was significantly higher in cases (1.0 mg/L) compared to controls (0.44 mg/L) (p=0.04). This could be caused by possible gastrointestinal and hepatic effects of chronic arsenic consumption, which could lead to inflammation and necrosis of the mucosa and submucosa of the stomach and intestine or cirrhotic portal hypertension (ATSDR 2007; Datta 1976). Experimental studies have also shown that arsenic exposure can inhibit endothelial nitric oxide synthase and produce inflammation and changes in coagulation, contributing to atherosclerosis (Simeonova and Luster, 2004). Case subjects also used tubewell water with a mean arsenic concentration that was 1.5 times higher than the water used by the controls (18.9 μg As/L compared to

12.3 μg As/L for controls; p=0.33) and had much higher As toenail levels compared to controls (3.5 μg As/gram toenail vs 1.8 μg As/ gram toenail; p=0.00). There was a significant trend among subjects who used water with the highest As level and As-associated skin lesions. Of the 46 subjects that used tubewell water with As levels higher than the Bangladeshi safety standard (>50 μg As/L) 63% were cases and 37% were controls (p=0.01). Case subjects were also more likely to have anemia (p=0.02), but not iron depletion (p=0.83). Breton et al found that higher hemoglobin levels were significantly protective against the presence of skin lesions in males, but not in Bangladeshi females (Breton et al. 2006). In our study, we found that women with As-associated skin lesions were 3 times more likely to have anemia (p=0.02) and vice-versa--women with anemia were 5.7 times more likely to have As-associated skin lesions (p=0.004).

The etiology of much of the anemia in our study population does not appear to be related to iron status, contrary to our expectations. Another possible cause of anemia could be deficiency of other nutritional factors that play a role in arsenic metabolism. Inadequate intakes of folate, methionine, calories, or protein are associated with arsenic-related health effects in both humans and animals (Vahter and Marafante, 1987; Hoffman et al, 1992; Lammon and Hood, 2004; Mitra el at , 2004; Chen et al, 1988). Other nutrients involved in one-carbon metabolism have been associated with the presence of skin lesions or with change in urinary arsenic species (Mitra et al, 2004; Steinmaus et al, 2005). Dietary factors that are related to one-carbon metabolism include folate,

methionine, cysteine, choline, betaine, and vitamins B_6 and B_{12} (Gamble et al, 2007). Gamble et al found that urinary DMA was positively associated with plasma folate, and negatively associated with homocysteine; they also found that inorganic arsenic and MMA were negatively correlated to plasma folate levels (Gamble et al, 2005).

Folate and other nutrients directly linked to one-carbon metabolism may be directly linked to the anemia found in our study population, as the prevalence of hyperhomocysteinemia is reportedly high in this area, indicating the folate in this population may be compromised, possibly resulting in megaloblastic anemia, rather than iron deficiency anemia (ACC/SNC, 2001). Additional blood parameters, such as the mean cell volume (MCV), would be helpful in distinguishing among the causes of anemia.

9.1 Strengths and Limitations

An important strength of our current study is that our population was homogeneous in terms of race, and age was evenly distributed across cases and controls. We also used toenail arsenic levels as a marker of long-term arsenic exposure. This is the first study that examines the etiology of anemia in the population of Bangladeshi women of reproductive age using serum ferritin in addition to hs-CRP to account for inflammation.

Our main limitation is the limited amount of serum volume we received, which precluded assessment of other biomarkers, such as folate, vitamin B_{12} and transferrin receptor. It would have been ideal to include transferrin receptor as one of our assessment tools for iron status, since this biomarker is reflective of iron stores and is not affected by infection or inflammation. These two conditions can be significant interfering factors in Bangladesh due to its high prevalence of communicable diseases. Although elevated CRP can identify some persons with inflammation, it has a faster spike than ferritin and a shorter half-life, so we may not have been able to identify all women who were actually iron deficient despite "normal" ferritin as a result of inflammation. .

Lack of quality dietary data is also a weakness, as we were not able to fully assess the relationship between food intake and iron status or arsenic toxicity. Due to lack of health data, such as parity, menstrual characteristics, infections we were not able to fully explore female-related factors that can influence iron status in this population, leaving room for potential residual confounding.

9.2 Future Directions

In order to test the validity of our conclusions, the next steps would include assessing plasma folate and vitamin B_{12}, in addition to observing the size of the red blood cells in the subjects with anemia (but not iron deficiency). Assessing hemoglobin from whole blood would be useful to get a more accurate assessment of their hemoglobin (anemia) status as well. Measuring transferrin

receptor would be the ideal step to get a more accurate determination of the prevalence of iron depletion in the presence of inflammation.

It would also be helpful to have more detailed dietary data, including water consumption and use (both frequency and duration of water use) to determine the extent to which whether arsenic toxicity results from exposure via water or food. Finally, assessment of water iron in addition to arsenic content from the tubewells where the subjects get their water would help to confirm whether drinking water helps protect against iron deficiency.

9.3 Conclusion

The prevalence of anemia was much lower than expected (18%) compared to previous estimates of 45% for the Bangladeshi female non-pregnant population and over 70% for the overall population (WHO, 2008; Ahmed, 2000). The prevalence of IDA was 4.8%. Women with As-associated skin lesions were more likely to have anemia, but not ID, compared to controls. The proportion of women with ID was much lower than expected (18%); however, once inflammation was accounted for, the prevalence increased to 28%. Women exposed to higher levels of water arsenic and those with higher toenail arsenic concentrations were less likely to have iron depletion. A possible explanation for this association could be the moderately strong correlation between arsenic and iron found in the tubewell water near our study region. It appears that a large proportion of the anemia observed in this population may be due to inadequacy of other

micronutrients, such as folate and B_{12}, which are needed in both hematopoiesis as well as arsenic metabolism. Further studies are needed to determine the folate and B_{12} status in women of reproductive age in Bangladesh, as these nutrients, which are also key players in pregnancy and reproductive health, may be compromised due to arsenic exposure.

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