

〔Biomarkers of Arsenicosis〕

A biomarker is defined as a xenobiotically induced alteration in cellular or biochemical components or processes, structures or functions, which is measurable in a biological system or sample (Marchiset-Ferlay et al., 2012). The selection of biomarker depends on reliability, less invasive sample as well easy to obtain are considered.

Diagnosis of arsenicosis is a difficult one. Traditionally it is based on the history of arsenic exposure, clinical feature(s), and presence of arsenic in hair, nail, or urine. If the patient is from an arsenic endemic area and has clinical feature of non-malignant skin manifestations, then it is easy to diagnosis it. On the other hand, a diabetic patient with arsenic in urine is difficult to establish it as a case of arsenicosis. We will face this problem until a biomarker specific for arsenicosis is identified.

Other biomarkers for arsenicosis such as micronuclei, sister chromatid exchange and HPRT mutant frequency have been described but they are not specific for arsenicosis (Table 4.1). Serum CC16 shows promise as a biomarker for assessing early respiratory damage induced by arsenic. There is an association between As^{III} methyltransferase polymorphism and arsenic-induced skin lesions (Valenzuela et al., 2009).

Table 4.1 Possible biomarkers of arsenicosis.

Sample	Biomarker
Hair	Arsenic
Nail	Arsenic
Blood	Malondialdehyde, metallothionein, micronuclei, sister chromatid exchange, HPRT mutant frequency, thioredoxin1, CC16, arsenic
Urine	Arsenic, malondialdehyde, 8-hydroxy-2'-deoxyguanosine, N-acetyl-β-D-glucosaminidase, microalbumin, retinol binding protein, β ₂ -microglobulin, transferrin, porphyrin, coproporphyrin-III
Oral mucosa cell	Micronuclei
Urothelial cell	Micronuclei

Scalp hair: Hair is rich in α-keratin that contains abundant cysteine residues (10-14%) to which arsenic binds and thereby accumulates arsenic. The concentration of arsenic in the scalp hair of normal individual is in the range of 0.02-0.2 µg/g of hair (Valentine et al., 1979; Olguin et al., 1983). Arsenic deposition in hair begins within 2 weeks of exposure and remains in these tissues for the next 1-2 years of life (Madorsky, 1977).

Arsenic exposure must be considered when its level is >0.5 µg/g of hair. This concentration of arsenic in arsenicosis is increased up to 10 µg/g of hair (Das et al., 1995). Presence of high concentration of arsenic in hair indicates recent or past exposure to arsenic. The arsenic level in hair of the patients of blackfoot disease is significantly higher than that of the controls, but still below the critical value of 1 µg/g (Lin & Yang, 1988). Sometimes the normal value of arsenic in hair does not exclude the diagnosis. Animal experiments have shown

that ingested organic arsenic (arsenobetaine and arsenocholine) is not deposited in hair (Vahter et al., 1983).

Speciation of arsenic is also examined. Scalp hair mainly contains As^{III} (60.9%) and As^{V} (33.2%). Others are MMA^{V} (2.2%) and DMA^{V} (3.6%), but no DMA^{III} (Mandal et al., 2003). No organic arsenic is accumulated in the hair following ingestion of fish arsenic.

Advantages – The use of scalp hair is a non-invasive method, easy to collect the sample and transport it from the field to the laboratory without any preservative.

Disadvantages – External contamination of arsenic is an important factor. When a person washes his/her hair with arsenic contaminated water without drinking shows high concentration of arsenic in hair (Harrington et al., 1978). There may lead to false positive result. Some patients do not prefer to give the sample due to superstition. Patient looks odd after proper collection of hair from the head so that the patient has to remove either all the hair after giving the sample or wear a cap on the head. In case of male patient, it may be done but in female, it is difficult to perform.

Nail: Like scalp hair, fingernail or toenail is rich in α -keratin that contains abundant cysteine residues (up to 22%). The concentration of arsenic in the nail of normal individual is 0.02-0.5 $\mu\text{g/g}$. Like scalp hair, arsenic deposition in nail begins within 2 weeks of exposure and remains in these tissues for the next 1-2 years of life (Madorsky, 1977). Arsenicosis must be considered when its level is $>1.0 \mu\text{g/g}$ of nail which is double the value of scalp hair. Presence of high concentration of arsenic in nail indicates recent or past exposure to arsenic. Like scalp hair, the normal value of arsenic in nail does not exclude the diagnosis.

Fingernail contains As^{III} (58.6%), As^{V} (21.5%), MMA^{V} (7.7%), DMA^{III}

(9.2%), and DMA^V (3.0%) (Mandal et al., 2003).

Advantages – The advantage of using nail is its slow growth rate, high affinity of arsenic for keratin, relatively easy to collection, storage and transport to the laboratory from the field without any preservative. It is a non-invasive method and is more preferable than scalp hair due to comparatively less chance of external contamination with arsenic. Toenail is more preferable than fingernail due to comparatively less chance of external contamination. Normal level of arsenic is a bit higher that is helpful for the less sensitive method of estimation. Unlike hair, there is no superstition of giving nail sample by the patient.

Disadvantages – External contamination of arsenic may cause false positive result. Time is needed to grow the nail, particularly toenail in order to collect it.

Urine: The normal level of total arsenic in blood is <1 µg/L in an un-exposed person. Urine is used not only for estimation of total arsenic but also for speciation. The presence of arsenic in urine indicates recent exposure of arsenic. Patients of blackfoot disease not only excrete arsenic in their urine but also mercury and lead. However, the urinary zinc and selenium are significantly lower in blackfoot disease than those of the normal controls (Horng & Lin, 1997; Lin & Yang, 1988).

Advantages – It is a non-invasive method and easy to collect. High concentration of arsenic in urine is useful for monitoring ongoing exposure.

Disadvantages – Special precaution is necessary for transport from the field level to the laboratory. There is lack of uniformity in sampling. 24-hours urine collection is sometimes difficult when the sample size is large. The main drawback with spot urine sample is the variation in dilution due to differences in the state of hydration, linked to fluid intake, physical activity and atmos-

pheric temperature (Marchiset-Ferlay, 2012). That is why, spot urine may not give the true result. Several confounding factors like age, sex, diet may influence the conclusion. For example, presence of arsenic of fish origin must be excluded if the urine level is used to identify possible toxicity (Hindmarsh, 2002). Fasting condition also affects the level of arsenic in urine. The percentage of urinary MMA level is found to be significantly increased after fasting (Brima et al., 2007). The time elapsed between the collection of urine and its analysis for arsenic is also important for correct estimation.

Blood: The normal level of total arsenic in blood is $<1 \mu\text{g/L}$ in unexposed person. It is used not only for the estimation of total arsenic, but also for speciation, malondialdehyde, metallothionein, micronuclei, sister chromatid exchange, HPRT mutant frequency, thioredoxin1 and CC16 levels. Patients of blackfoot disease showed significantly lower concentrations of selenium and zinc in the blood than the normal control (Lin & Yang, 1988). The amount of arsenic in blackfoot disease is about 60 ppb. It is present more in the red blood cell (98 ppb) than in plasma (38 ppb) (Heydorn, 1970).

Very low amount of arsenic is present in blood in individual even exposed to high concentration of arsenic.

Advantages – Blood arsenic is typically used as an indicator only in the case of very recent exposure or relatively high-level exposure following acute arsenic poisoning.

Disadvantages – It is an invasive method. However, low-level of inorganic arsenic exposure from drinking water and organic arsenic from food are difficult to distinguish, thus causing a limitation of blood arsenic levels as indicators (NRC, 1999). Therefore, a more sensitive method of estimation is required.

Micronuclei: The formation of micronucleus is one of the most sensitive biomarkers of chromosomal damage and genome stability in human populations (Figure 4.1) (Moore et al., 1997). The frequency of micronuclei in the arsenic exposed people is significantly elevated to 5.33-fold over unexposed levels for lymphocytes, 4.63-fold for oral mucosa cells, and 4.71-fold for urothelial cells (Basu et al., 2004). Among these three cell types, slightly higher level of micronuclei being observed in lymphocytes compared with oral mucosa and urothelial cells.

Usefulness of micronuclei assay as a screening and early detection technique for cancer susceptibility has been suggested. The normal mammalian cell culture derived from male Chinese hamster lung fibroblast cells (V79) was used as the test system to assess the genotoxicity by micronucleus assay. The results showed that both green tea and black tea extracts have equal potentiality in modulating the arsenic-induced genotoxicity (Sinha et al., 2005).

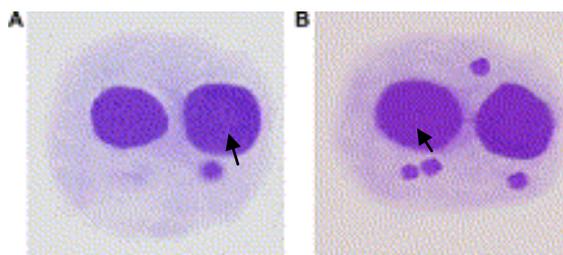


Figure 4.1 Representative images of micronuclei formation (arrow) in erythrocytes of *Tilapia* fish after exposure to arsenic (NaAsO_2) for 96 hours; (A) moderate micronucleus formation at 3 ppm and (B) severe micronucleus formation at 56 ppm of exposure respectively.

Several confounding factors like lifestyle (exercising, drinking, and smoking), dietary (folate deficiency, plasma levels of vitamin B_{12} and homocysteine) and demographic factors (age and gender) can influence the formation and the frequency of cellular micronuclei (Ishikawa et al., 2003).

Sweat: The skin of healthy control secretes very low amount of arsenic (mean \pm SE; unit: $\mu\text{g}/\text{inch}^2$ of skin/24 hours; chest: 0.6 ± 0.2 ; back: 0.3 ± 0.1 ; and abdomen: 0.5 ± 0.2) (Yousuf et al., 2011). Several folds higher amount of arsenic is secreted in people exposed to arsenic. Highest amount is found in the chest for arsenic-exposed controls (8.4 ± 1.8) and patients (9.2 ± 1.3), respectively). The lowest amount is secreted in the abdomen of arsenic-exposed control (6.5 ± 1.7) and patient (5.2 ± 1.0), respectively). No significant differences are observed between the arsenic-exposed control and patient in arsenic secretion through skin.

E. coli in stool: Arsenic exposure decreased the colony count of *E. coli* in stool. It is severely reduced in the stool of patients. In addition, excretion of arsenic in the stool of arsenic exposed control and patient is increased. However, patients show less excretion of *E. coli* in stool than arsenic exposed controls (Rashid et al., 2014).

Sister chromatid exchange: Sister chromatid exchange means the exchange of genetic materials between the two identical sister chromatids. Four to five sister chromatid exchange per chromosome pair per mitosis is in the normal distribution. Frequent sister chromatid exchange may also be related to the formation of tumor. As^{III} induces a significantly increased frequency of sister chromatid exchange in Chinese hamster and Syrian hamster embryo cells. As^{V} is one order of magnitude less potent in inducing sister chromatid exchange than As^{III} .

Hypoxanthine guanine phosphoribosyltransferase (HPRT) gene: HPRT is an enzyme encoded in humans by the HPRT1 gene. It catalyzes the conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate. HPRT gene is located on the X chromosome. Study was conducted to investigate the feasibility of using mutagenesis of the hypoxanthine guanine

phosphoribosyl transferase (HPRT) gene in T-lymphocytes as a quantitative biomarker for detection of biological damage caused by arsenic (Harrington-Brock et al., 1999). The HPRT T-cell assay does not appear to have sufficient sensitivity to be useful as a biomarker of genetic effects caused by low-level arsenic exposure.

Metallothionein: Metallothionein is a low molecular weight (500 to 14,000 Da) metal-binding protein that protects our body against metal intoxication. It is rich in cysteine. Blood metallothionein level is significantly lower in arsenicosis in Guizhou as compared to control (Liu et al., 2007). Metallothionein has the capacity to bind zinc, copper, selenium, cadmium, mercury, silver, arsenic through the thiol group of its cysteine residues. So, metallothionein level is not specific for arsenicosis.

Porphyrin: The well-known porphyrin is the heme. Coproporphyrin is the metabolite arising from heme synthesis. The major abnormalities in the urinary porphyrin excretion pattern observed in arsenic-exposed individuals are: a) significant reductions in coproporphyrin III excretion resulting in decrease and b) significant increase in uroporphyrin excretion (García-Vargas et al., 1994). Both alterations are responsible for the decrease in the COPRO/URO ratio. No porphyrinogenic response is found in individuals with urinary arsenic concentrations (<1,000 µg arsenic/g creatinine). A study in the Guizhou Province, China, found significantly raised levels of urinary uroporphyrin-III and coproporphyrin-III (but not coproporphyrin-I) in the arsenic-exposed group as compared to control (Deng et al, 2007).

Malondialdehyde: The level of malondialdehyde in plasma, erythrocyte or urine may be used as a biomarker of oxidative stress. The level of urinary malondialdehyde in arsenicosis (arsenic contaminated by coal burn) is increased which indicates that arsenic exposure causes oxidative stress (Wang et al.,

2009). This biomarker is not specific for arsenic.

Reduced glutathione: Arsenicosis shows low level of reduced glutathione (GSH). The mean GSH level in red blood cells in arsenicosis is 55.3 mg/dL, in arsenic exposed family members is 57.8 mg/dL, and in the normal control group is 88.7 mg/dL (Sinha et al., 2003).

8-Hydroxy-2'-deoxyguanosine (8-OHdG): The oxidative damage permanently occurs to lipids of cellular membranes, proteins, and DNA. In nuclear and mitochondrial DNA, 8-OHdG is one of the predominant forms of free radical-induced oxidative lesions, and has, therefore, been widely used as a biomarker for oxidative stress and carcinogenesis. Urinary 8-OHdG shows a significant dose-response relationship after 8 months of exposure of arsenic. This marker is also not specific.

Studies were done to find out an effective biomarker of kidney toxicity caused by exposure to arsenic. These studies are focused on N-acetyl- β -D-glucosaminidase, β_2 -microglobulin, microalbumin and retinol binding protein (Buchet et al., 2003).

Microalbumin: α_1 -Microglobulin is one of the three original members (α_1 -microglobulin, retinol binding protein and β -lactoglobulin) of the lipocalin superfamily. Its function in blood and urine is not clear. However, it may play a biological role as an anti-oxidant with oxidant-scavenging and enzymatic reductase properties. α_1 -Microglobulin in medium and severe form of arsenicosis is significantly higher than that in the control group (Zhang et al., 2006).

Retinol binding protein: Retinol binding protein, a carrier protein of retinol, has diverse functions. Urinary retinol binding protein is the sensitive indicator of renal tubular damage. This protein is significantly higher in medium and severe form of arsenicosis than that in the control group (Zhang et al., 2006).

Transferrin: Transferrin (80,000 Da) is an iron binding protein in the blood that transports iron throughout the body. Its level rises in case of iron deficiency and fall in cases of iron overload. Transferrin level in severe arsenicosis is increased significantly compared with the control group (Zhang et al., 2006).

Heme oxygenase: Physiological degradation of heme to biliverdin is mediated by an enzyme heme oxygenase (31,000 Da). This enzyme is present within the macrophage. Biliverdin is converted to bilirubin by biliverdin reductase. Bilirubin is then enter into the intestine through bile duct. Bilirubin is converted to urobilinogen (stercobilinogen) by removing glucuronic acid by bacteria within the intestine (Figure 4.2). Some amount of urobilinogen is converted to stercobilin and others enter into the liver via portal vein (enterohepatic circulation).

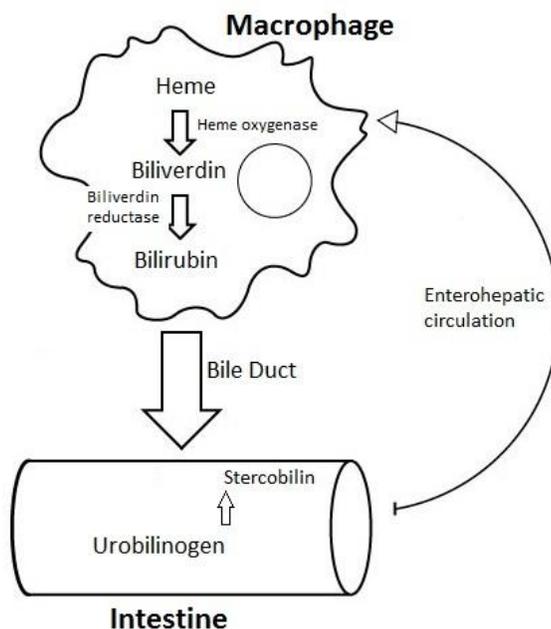


Figure 4.2 Role of heme oxygenase in heme metabolism.

The activity of heme oxygenase is increased up to 100-fold by a wide variety

of stimuli (metals, heme and hormones). It is also increased in arsenicosis which may be due to arsenic (Del Razo et al., 2001). Therefore, induction of heme oxygenase is not specific for arsenicosis.

Thioredoxin: Thioredoxin is a protein (12,000 Da) that acts as an antioxidant by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. There is a significant increase in the concentrations of serum thioredoxin1 in low, medium, high arsenic exposure groups, and the arsenicosis group, in a dose-response manner (Li et al., 2012). Thioredoxin might play an important role in the methylation of arsenic by the enzyme arsenic III methyltransferase due to the fact that serum thioredoxin1 level appears to be necessary for the catalysis of arsenic methylation (Thomas et al., 2004; Waters et al., 2004).

4.1 Collection of Sample

Water: Study participant is provided with acid-washed (nitric acid-water, 1:1) plastic bottle (50 mL size) for the collection of drinking water sample into which nitric acid (1.0 mL/L or 1 drop/50 mL bottle) is added later on as a preservative.

Blood: Whole blood, serum, plasma and erythrocytes are collected from the venous blood.

Urine: First morning void is collected in precoded polypropylene bottle (50 mL size) for arsenic estimation as this gives the best measure of recent arsenic exposure. Immediately after collection, the sample is stored and carries to the laboratory at 0 °C at ice box and then is kept at – 20 °C until estimation is carried out. Concentrated HCl (1 mL/100 mL urine) is added in the urine sample to prevent bacterial growth. Urine sample should be filtered before analysis in

order to remove epithelial cells that may contain arsenic. This will help to get the exact result of the amount of arsenic in urine.

Nail: Ceramic blade cutter is used to collect nail sample. Use of normal blade may increase the amount of arsenic in nail. After collection, the sample is thoroughly cleaned by sonication with double distilled water followed by an acetone wash for 5 min to remove exogenous arsenic.

Hair: Ceramic blade cutter is used to collect scalp hair sample. Usual scissors is avoided for external contamination of arsenic. Sample is thoroughly cleaned like nail to remove exogenous arsenic. Hair sample is of similar size and is taken from more or less similar region of head (close to the scalp behind the ear with a diameter of about 1 cm) (Maki-Paakkanen et al., 1998). The selection of this area is due to less chance of external contamination. Another option is to collect hair sample of at least 1 g of hair from several sites on the head and a mean level should be taken (Hindmarsh, 2002).

Oral mucosa cell: Oral mucosa cell sample is collected from each subject using a soft toothbrush to scrape cells gently from the oral mucosa (inside of both cheeks). The brush is then swirled into a centrifuge tube containing a buffer solution containing EDTA (0.1 M), Tris-HCl (0.01 M), NaCl (0.02 M; pH 7.0) (Warner et al., 1994), thereby creating a cell suspension. The cell suspension is stored at 2-4 °C in a cooling device and brought to the laboratory within 2 hours of sample collection.

Urothelial cell: To collect urothelial-exfoliated cells, each subject is asked to provide 50 mL of the urine sample from the second and third voids of the day. The urine sample is coded, kept at 2-4 °C in a cooling device, and carries to the laboratory within 2 hours of sample collection. First morning void is not used for micronuclei assay because exfoliated cells tend to degrade from overnight

exposure to urine. Female generally exfoliates more cells per void than male.

4.2 Laboratory Analysis

Hair: Hair sample is used for the estimation of total arsenic or speciation. Estimation of total arsenic is usually done and requires less expensive equipment. Speciation of arsenic in hair is carried out by HPLC-ICP-MS. For each case, hair sample is digested with acids at 90 °C until the white fume comes out.

Nail: Estimation of arsenic in nail is similar to hair.

Micronuclei assay in exfoliated epithelial cells: Oral mucosa cells are obtained by simply centrifuging the cell suspension at 1,500 rpm for 10 min. The supernatant is discarded and cell pellets are resuspended in fresh buffer solution. Cells are washed thrice with the buffer solution. Gentle pipetting of cells in the buffer solution reduces clumping and lyse broken cells. Volumes of 25 mL of the buffer solution in 50 mL conical tubes are used in every washing step.

Urothelial cells: Urothelial cells are recovered by centrifuging urine samples (2,000 rpm for 15 min) and washing the cell pellet with 0.9% NaCl. Cell suspension of both cell types (50 µL) is laid and spread well on clean, preheated (40 °C) glass slides and allows to air-dry for 5-10 min. Cell density is checked with a phase-contrast microscope. The cell solution is either concentrated by centrifugation or diluted in the buffer solution (for oral mucosa cells) or 0.9% NaCl (for urothelial cells) as required. Once the desired cell density (no overlapping cells) is reached, more slides are prepared. The slides are fixed in methanol (80% v/v) at 0 °C for 20 min and air-dried.

Micronuclei in oral mucosa cells are scored in accordance with the criteria reported (Tolbert et al. 1992), while urothelial cells are analyzed by the method

described (Reali et al. 1987). At least 3,000 oral mucosa cells and 1,000 urothelial cells are scored per individual.

Micronuclei assay in lymphocytes: Lymphocyte cultures are carried out for micronuclei analysis following the protocol (Fenech, 1998). The whole blood cultures are incubated for 44 hours at 37 °C. Cytochalasin B is added to each culture to give a final concentration of 6 µg/mL and the culture is incubated at 37 °C for an additional 28 hours to induce binucleated cell formation. After a total of 72 hours incubation, the cells are centrifuged at 1,000 rpm for 5 min. Supernatant is discarded and cell pellets are treated with 0.075 M KCl/saline (1:9) for 5 min. After centrifugation, the cells are fixed in fresh fixative (methanol/glacial acetic acid, 3:1). Fixative is removed by centrifugation and two more changes of fixative are performed. The cells are dropped onto wet clean slides and the slides are air-dried and stained with 5% Giemsa in phosphate buffer (pH 6.8). Finally binucleated cells from each subject are examined for micronuclei under the microscope.

Scoring procedure – All slides are first examined with low-power (20x) magnification using a compound microscope to discard those infected with bacteria, fungi, and polymorphonuclear leukocytes as these may interfere with scoring. Slides are then scored at 100x (oil immersion lens). Smear, clumped overlapped or necrotic cells or those without intact nuclei are not recorded. Only those micronuclei are noted which are a) rounded or oval shaped; b) less than one-third the diameter of the main nucleus; c) in the same focal plane as the nucleus; d) of the same color, texture, and refraction as the main nucleus; and e) clearly separated from the main nucleus.

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