

INSTITUTE OF ENVIRONMENTAL MEDICINE
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**FACTORS INFLUENCING
THE METABOLISM OF
INORGANIC ARSENIC IN
HUMANS**

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LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
AAS	Atomic absorption spectrometry
AB	Arsenobetaine
AC	Arsenocholine
AFS	Atomic fluorescence spectrometry
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APL	Acute promyelocytic leukemia
As(III)	Arsenite
As(V)	Arsenate
AS3MT	Arsenic (III) methyltransferase
BMI	Body mass index
CCA	Copper chrome arsenate
DMA	Sum of dimethylarsinite and dimethylarsinate
DMA(III)	Dimethylarsinite
DMA(V)	Dimethylarsinate
DNA	Deoxyribonucleic acid
ERKs	Extracellular signal-regulated kinases
GSH	Glutathione
GSTM1	Glutathione-S-transferase mu 1
GSTO1	Glutathione-S-transferase omega 1
GSTT1	Glutathione-S-transferase theta 1
HDSS	Health and demographic surveillance system
HG	Hydride generation
HPLC	High performance liquid chromatography
IARC	International agency for research on cancer
iAs	Sum of arsenite and arsenate
ICDDR,B	International centre for diarrhoeal disease research, Bangladesh
ICPMS	Inductively coupled plasma mass spectrometry
ICPSFMS	Inductively coupled plasma sector field mass spectrometry
MA	Sum of methylarsonite and methylarsonate
MA(III)	Methylarsonite

MA(V)	Methylarsonate
MAPKs	Mitogen-activated protein kinases
MTHFR	Methylene tetrahydrofolate reductase
MTR	5-methyltetrahydrofolate homocysteine methyltransferase
NRC	US National research council
PCR	Polymerase chain reaction
PNP	Purine nucleoside phosphorylase
ROS	Reactive oxygen species
SAM	S-adenosyl methionine
SHBG	Sex hormone binding globulin
SH-groups	Sulfhydryl groups
SNPs	Single nucleotide polymorphisms
SumAs	Sum of arsenic metabolites
WHO	World health organization

Magnus (1193-1280). Around 1250 he heated orpiment with soap and formed nearly pure arsenic.

1.1.1.1 Poison

Arsenic is one of the most notorious poisons of all times. The presence of arsenic (in form of As_2O_3) in lethal doses in food, water, or air are not noticeable to human senses of sight, smell and taste, which makes it a perfect homicidal and suicidal agent. The Swedish king Erik XIV died in 1577 after spending over 9 years in a dungeon at the Royal Palace in Stockholm. Rumors that he had been poisoned by his brother Johan III spread immediately, and almost 400 years later it was confirmed that he had been poisoned by arsenic (Vahter, 1983). The death of the French emperor Napoleon Bonaparte in 1821 has also been believed to be due to arsenic poisoning. Indeed, high arsenic concentrations have been found in hair samples taken from his head (Kintz et al., 2006), but this is still a controversial issue (Keynes, 2004).

Because of As_2O_3 being taste-, odor- and colorless a lot of accidental arsenic poisonings have occurred throughout history. Arsenic poisoning was a problem in Victorian-era England, mainly because arsenic was used as dyes and pigments in several consumer products, including wallpaper, toys, food wrappers and clothing made from dyed fabrics. The most frequently used arsenic compound was copper arsenite, also known as Scheele's Green. Copper arsenite was, under humid conditions, converted into gaseous di- and tri-methylated arsines by mold living in wallpaper paste and caused chronic poisoning and even fatalities (Meharg, 2005). In Manchester in 1900-1901 around 6,000 people, with 80 fatalities, were poisoned from drinking arsenic contaminated beer. It was found that the sugar used in the production of the beer was prepared by hydrolyzing starch with arsenic-contaminated sulfuric acid. Accidental mass poisonings have also occurred in modern times. In 1955, over 12,000 Japanese infants were poisoned by dried milk made with arsenic-contaminated sodium phosphate as a stabilizer (Dakeishi et al., 2006).

1.1.1.2 Medicine

The therapeutic use of arsenic dates back to 400 B.C. with Hippocrates recommending arsenic for treatment of ulcers. Most commonly used were simple compounds such as the two sulfides, realgar and orpiment. The peak of the use as a therapeutic agent took place during the 19th century after the introduction of "Liquor Mineralis", consisting of

1% potassium arsenite, in 1786 by Thomas Fowler. It was widely prescribed for various dermatoses such as psoriasis and syphilis, infectious diseases, epilepsy and asthma (Meharg, 2005). Almost a century ago the Nobel Prize winner Paul Ehrlich together with Sakahiro Hata discovered the effectiveness of arsphenamine, also known as Salvarsan, in the treatment of syphilis. Arsphenamine was soon followed by neoarsphenamine, which was water soluble and therefore easier to administer. These drugs remained the main treatment against syphilis for forty years, until penicillin rendered them obsolete (Thorburn, 1983).

1.1.2 Current use

The largest part of the world's arsenic production is used in timber treatment as copper chrome arsenate (CCA, almost 70%) and the second largest part is used as agricultural chemicals (WHO, 2001). In the electronics industry there is an increased use of gallium arsenide and alloys in semiconductors. Glass industries in many countries still use arsenic trioxide as a clarifier (Peters et al., 1996).

The use of arsenic as a pharmaceutical has been resumed in the past years. Arsenic trioxide (As_2O_3) has been shown to be effective in the treatment of acute promyelocytic leukemia (APL) with its ability to induce apoptosis and differentiation of the cancer cells at rather high concentrations (Lallemand-Breitenbach et al., 2005; Zhu et al., 2002). The standard dosage for human patients with APL is 0.16 mg As/kg over a period of six weeks (Niu et al., 1999; Shen et al., 2004). Further research on anti-cancer activity of arsenic and its combination with other treatments, e.g. retinoic acid, is ongoing.

1.1.3 Occurrence in the environment

1.1.3.1 Natural sources

Arsenic occurs naturally in many mineral ores and is the main constituent of more than 200 mineral species. The most common mineral is arsenopyrite. Arsenopyrites are under certain conditions oxidized to yield arsenic containing salts that are readily water soluble and could leach out into the groundwater. Arsenic also occurs in sedimentary deposits (WHO, 2001). Elevated concentrations of arsenic in groundwater used as drinking water have been found all over the world, e.g. Taiwan (Chen et al., 2005a), India (Rahman et al., 2005), Bangladesh (Rahman et al., 2006b), China (Pi et al., 2002), Chile (Caceres et al., 2005), Mexico (Meza et al., 2004), Argentina (Vahter et al.,

1995a) and Romania (Aposhian et al., 2000). In Bangladesh alone over 50 million people drink water containing more arsenic than the World Health Organization (WHO) guideline of 10 µg/L (Chakraborti et al., 2004; WHO, 2003). Surface and sea water, however, are generally very low in arsenic, typically 1-2 µg/L (WHO, 2001). Seafood and algae may contain high concentrations of arsenic (in the mg/kg range; Francesconi and Kuehnelt, 2002), although, the forms present are mainly organic arsenicals, e.g. AB, AC and arsenosugars, with low toxicity and rapid excretion into urine (Marafante et al., 1984; Sabbioni et al., 1991; Vahter et al., 1983).

1.1.3.2 Anthropogenic sources

The largest anthropogenic contributor to arsenic release into the environment is the metal production (mining and smelting) industry. Agricultural use and the burning of fossil fuels follow as the next most important anthropogenic sources.

1.2 ASSESSMENT OF EXPOSURE AND ANALYTICAL METHODS

When assessing the exposure to arsenic the most common measurement used is arsenic concentrations in drinking water, as it is not always feasible to collect urine or other biological samples from a large number of individuals. However, due to the variation in water intake, the actual ingested amount of arsenic is difficult to evaluate based on the water concentration (Calderon et al., 1999). Individual exposure to arsenic is better assessed by using biomarkers of exposure. There are several biomarkers for arsenic exposure, e.g. arsenic in blood, urine, hair and nails (Chen et al., 2005a). Urine is the most common for evaluation of recent arsenic exposure as urine is the major route of excretion and samples are easy and non-invasive to collect. A problem, however, is that spot urine samples (single voided urine) are often used, as complete 24-hour urine, which is the preferable standard, is very difficult to collect as it is highly inconvenient for the subjects under study. The problem with spot urine samples is the variation in dilution due to variation in the intake of fluids, physical activity, temperature etc (Suwazono et al., 2005). Therefore, it is essential to adjust for this variation in dilution when comparing concentrations between individuals and populations. The commonly used approach to handle this problem is to relate the arsenic concentration in urine to the urinary creatinine excretion, which is fairly constant in healthy individuals. Another approach is to adjust the arsenic concentrations for the specific gravity of the urine (Nermell et al., 2007).

Furthermore, as a single meal of seafood may increase total urinary arsenic concentrations several orders of magnitude, total arsenic in urine is not a reliable indicator of exposure to inorganic arsenic (Vahter, 1994). Therefore, it is important to distinguish between species derived from exposure to inorganic arsenic and species derived from organic arsenic exposure, e.g. AB and AC. Exposure to inorganic arsenic is often assessed by measuring either the separate species, i.e. As(III), As(V), methylarsonate (MA(V)) and dimethylarsinate (DMA(V)), or the sum of these species in urine (which henceforth will be referred to as SumAs).

Several commercial field test kits are available on the market. They are commonly used when analyzing arsenic in drinking water, due to its simplicity and low costs (Jakariya et al., 2007; Rahman et al., 2002; Steinmaus et al., 2006b; Van Geen et al., 2005). However, these have been shown to only be semi-quantitative with poor accuracy, especially at concentrations below 50 µg/L. The most frequently used laboratory method for determination of arsenic in drinking water is atomic absorption spectrometry (AAS), with its low detection limits and high accuracy (Wahed et al., 2006). For determination of inorganic arsenic and its metabolites in urine some commonly used analytical methods include speciation analysis with high performance liquid chromatography (HPLC), with or without hydride generation (HG), coupled to inductively coupled plasma mass spectrometry (ICPMS) or atomic fluorescence spectrometry (AFS). Determination of SumAs with AAS coupled to HG is also a frequently used method (Francesconi and Kuehnelt, 2004; Norin and Vahter, 1981). Hydride generation is commonly coupled to the detector to discriminate for the organic arsenic species AB and AC as these do not form volatile arsines like inorganic arsenic and its metabolites do.

1.3 TOXICITY

This chapter will focus on the toxic effects after chronic exposure to inorganic arsenic, as acute exposure is not relevant in the context of this thesis.

1.3.1 Toxic effects

Carcinogenic effects of arsenic compounds were recognized more than 100 years ago by Hutchinson (Hutchinson, 1887). The International Agency for Research on Cancer (IARC) first evaluated the carcinogenic effect of arsenic in 1973 and concluded that arsenic causes skin cancer in people exposed to inorganic arsenic both in the working

environment and via drugs, drinking water and pesticides. It was concluded, however, that there was not sufficient evidence that inorganic arsenic was carcinogenic in experimental animals (IARC, 1973). The same conclusions were drawn in the second evaluation performed in 1980 (IARC, 1980). In the most recent evaluation, inorganic arsenic was classified to be carcinogenic to humans (Group 1) and causes cancer of the urinary bladder, lung, skin and possibly also kidney and liver (IARC, 2004).

Evidence that inorganic arsenic is carcinogenic in animals have been lacking for a long time. However, recently several studies in experimental animals have demonstrated the carcinogenic effect of arsenic species in the same organs as in humans (Kitchin, 2001; Waalkes et al., 2007; Waalkes et al., 2006; Wang et al., 2002). The lack of positive carcinogenic data in animal studies in the past could be due to the opposing effects of inorganic arsenic at different doses. As previously discussed As_2O_3 is used in APL treatment due to its ability to inhibit tumor growth and angiogenesis at high concentrations (Liu et al., 2006). Another reason for the negative carcinogenic data could be the extensive metabolism to DMA by most experimental animals, which in turn is rapidly excreted (Vahter, 1999a).

The National Research Council (NRC) has recently performed risk assessment of arsenic in drinking water and conclude that the cancer risk at water concentrations of 10 $\mu\text{g/L}$, the guideline recommended by the WHO (WHO, 2003), far exceed the tolerable limit of 1 extra cancer case per 100,000 individuals (NRC, 1999; NRC, 2001). For bladder cancer the excess lifetime risk was calculated to be 12 for women and 23 for men per 10,000 individuals and for lung cancer the risk was calculated to be 18 and 14 per 10,000 individuals for men and women, respectively.

The earliest signs of toxicity from chronic exposure to inorganic arsenic in humans are pigmentation changes. However, the latency period is normally about 5-10 years (NRC, 1999; NRC, 2001). Hyperkeratosis usually follows the initial pigmentation changes and could proceed to skin cancer. Two different types of arsenic induced skin cancers are seen, basal cell carcinoma, which is usually only locally invasive and squamous cell carcinoma, which may have distant metastases. The arsenic induced skin cancers differ from those induced by ultraviolet light by generally occurring on areas of the body not exposed to sunlight, e.g. soles and palms (Mazumder et al., 1998; NRC, 1999; NRC, 2001; Rahman et al., 2006b). Other non-carcinogenic effects induced by arsenic

include vascular diseases, liver- and neurotoxicity, chronic cough and diabetes mellitus (Mazumder et al., 2000; WHO, 2001). Recent studies have also seen associations between arsenic exposure and adverse pregnancy outcomes and impaired child development (Rahman et al., 2007; Wasserman et al., 2007).

1.3.2 Mechanism of toxicity

Although a large amount of research has been performed on the mechanism of arsenic toxicity, the exact nature of the carcinogenic effect of arsenic is not yet clear. The valence state and form of arsenic are of great importance for the toxicity. The trivalent forms of arsenic are the principal toxic forms, which react with e.g. enzymes and transcription factors (Hughes and Kenyon, 1998; Kligerman et al., 2003; Petrick et al., 2000; Petrick et al., 2001; Schwerdtle et al., 2003a; Schwerdtle et al., 2003b; Styblo et al., 2000; Styblo et al., 2002; Vega et al., 2001).

Several modes of action of the carcinogenic effect of arsenic have been proposed, e.g. genotoxicity, altered DNA repair, induction of oxidative stress, altered DNA methylation, altered cell proliferation and altered cell signaling. Arsenic can induce chromosomal aberrations, micronuclei, aneuploidy, endoreduplication and gene amplification (Chakraborty et al., 2006; Gebel, 2001; Huang et al., 1995; Mahata et al., 2003). Arsenic has been seen to inhibit several DNA-repair enzymes, especially zinc-finger proteins (Andrew et al., 2006; Li and Rossman, 1989; Schwerdtle et al., 2003a; Witkiewicz-Kucharczyk and Bal, 2006). Arsenic exposure results in the formation of reactive oxygen species (ROS) that could lead to DNA adduct formation like 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is used as a common marker of oxidative DNA damage (Ding et al., 2005; Fujino et al., 2005; Kessel et al., 2002; Kubota et al., 2006; Matsui et al., 1999). The alteration of DNA methylation may also be involved in arsenic carcinogenesis. Arsenic can cause hypo-methylation of DNA leading to altered gene expression, probably due to the inhibition of DNA methyltransferases (Chanda et al., 2006; Cui et al., 2006a; Reichard et al., 2007; Sciandrello et al., 2004). Signal transduction pathways have also been demonstrated to be a target for arsenic interaction. Mitogen-activated protein kinases (MAPKs) like extracellular signal-regulated kinases (ERKs) have been shown to be induced at low arsenic levels leading to uncontrolled cell proliferation and transformation (Bode and Dong, 2002; Qian et al., 2003).

A wide variation in susceptibility to arsenic induced health effects has been observed between human population groups and individuals (NRC, 1999; NRC, 2001). This has been suggested to, in part, be due to the large inter-individual variation in arsenic metabolism.

1.4 KINETICS

This chapter will focus on the kinetics after exposure to inorganic arsenic via drinking water and food. Occupational exposure will not be included, as this is a large area in itself with partly different kinetics and effects.

1.4.1 Absorption

The absorption of both As(III) and As(V) in the gastrointestinal tract is high in both humans and experimental animals, approximately 80-90% (Pomroy et al., 1980; Vahter and Norin, 1980). The dermal absorption, however, is very low (Wester et al., 1993). Several studies have demonstrated that urinary arsenic increase significantly after respiratory exposure in workers (Hakala and Pyy, 1995; Offergelt et al., 1992; Vahter et al., 1986; Yager et al., 1997; Yamauchi et al., 1989), indicating that arsenic is absorbed in the respiratory tract. However, it is not possible to quantify as oral and respiratory exposure were not distinguishable in these studies.

1.4.2 Biotransformation

The biotransformation of inorganic arsenic in most mammals, including humans, involves a series of reduction and methylation reactions, with DMA being the main metabolite found in urine (Buchet and Lauwerys, 1988; Challenger, 1945; Cullen et al., 1984; Hirata et al., 1990; Marafante et al., 1985; Thompson, 1993; Vahter and Envall, 1983; Vahter and Marafante, 1988).

The reduction reactions seems to occur already in the blood, using thiols as electron donors (Marafante et al., 1985; Vahter and Envall, 1983; Vahter and Marafante, 1985). More recent *in vitro* studies have shown that the reduction of As(V) could occur both enzymatically by reductases and non-enzymatically in the presence of thiols (NRC, 1999; NRC, 2001). Only one reductase has been identified in humans, namely glutathione-S-transferase omega (GSTO1; Zakharyan et al., 2001). A purine nucleoside phosphorylase (PNP) has been shown to catalyze the reduction *in vitro* (Gregus and

Nemeti, 2002; Radabaugh et al., 2002), but its *in vivo* relevance is not clear (Nemeti et al., 2003; Nemeti and Gregus, 2004).

The methylation mainly occurs in the liver, where the blood from the intestine passes through before being distributed to any other organ, but other tissues have also been shown to have methylating ability, but to a much lesser extent. (Buchet et al., 1984; Buchet and Lauwerys, 1985; Charbonneau et al., 1979; Geubel et al., 1988; Healy et al., 1998; Lerman et al., 1983; Vahter, 1981). The methylating activity is localized in the cytosol via the one-carbon metabolism (**Fig. 1**) with S-adenosyl methionine (SAM) as the main methyl donor (Buchet and Lauwerys, 1985; Marafante and Vahter, 1984; Styblo and Thomas, 1997; Zakharyan et al., 1995). One methyltransferase, that catalyze the methylation reactions, has been identified in humans, i.e. arsenic (III) methyltransferase (AS3MT; Lin et al., 2002).

Two pathways for the metabolism of inorganic arsenic have been suggested. The classical pathway suggests alternating reduction and oxidative methylation with trivalent metabolites, methylarsonite (MA(III)) and dimethylarsinite (DMA(III)), following the pentavalent metabolites, MA(V) and MA(V), resulting in only one end product, i.e. DMA(III) (Challenger, 1945; Cullen and Reimer, 1989). The second newly proposed pathway suggest the opposite, that the trivalent metabolites instead precedes the pentavalent metabolites, producing two end products, both MA(V) and DMA(V) (Hayakawa et al., 2005).

Considerable differences in arsenic metabolism have been observed between different mammalian species. Most studied animals are more efficient in methylating arsenic to DMA than humans, except the chimpanzee and the marmoset monkey which have been shown not to methylate arsenic at all (Vahter et al., 1995b; Vahter and Marafante, 1985; Vahter et al., 1982). Most experimental animals do not excrete a significant proportion of MA in urine as humans do. The rat differs from most mammalian species by accumulating DMA in erythrocytes, probably by binding to a cystein component in the hemoglobin (Lu et al., 2007).

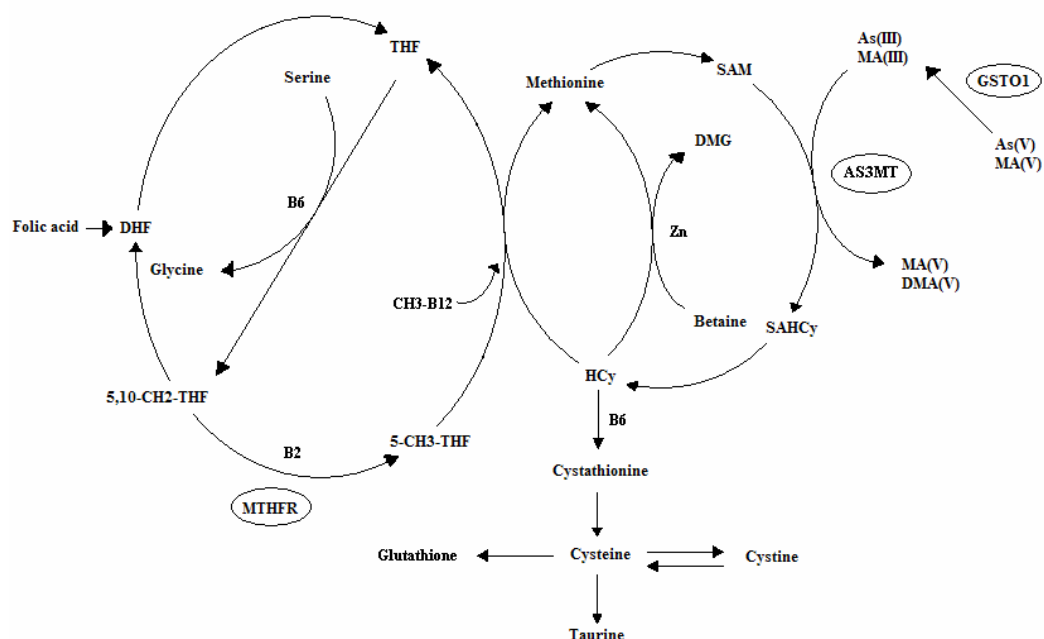


Figure 1. The one carbon metabolism and the metabolic pathway of arsenic. As(III), arsenite; As(V), arsenate; MA(III), methylarsonite; MA(V), methylarsonate; DMA(III), dimethylarsinite; DMA(V), dimethylarsinate; SAM, S-adenosyl methionine; SAHCy, S-adenosyl homocysteine; HCy, homocysteine; DMG, dimethylglycine; 5-CH₃-THF, methyl tetrahydrofolate; 5,10-CH₂-THF, methylene tetrahydrofolate; DHF, dihydrofolate; THF, tetrahydrofolate; *GSTO1*, glutathione-S-transferase omega; *As3MT*, arsenic (III) methyltransferase; *MTHFR*, methylene tetrahydrofolate reductase; B₂, vitamin B₂ (Riboflavin); B₆, vitamin B₆; CH₃-B₁₂, methylated vitamin B₁₂; Zn, zinc.

Large variations in arsenic metabolism, especially in the formation of MA, have also been seen between human population groups. In general, urine consists of 10-30% inorganic arsenic, 10-20% MA and 60-80% DMA, which are commonly used as a measure of methylation capacity or efficiency (Hopenhayn-Rich et al., 1993; Vahter, 2002). However, indigenous people in the north of Argentina only have a few percent of MA in urine (Vahter et al., 1995a), while people in Taiwan have 20-30% (Chiou et al., 1997; Hsueh et al., 1998).

Studies indicate that arsenic metabolism is of importance for the variation seen in susceptibility of arsenic induced toxicity. A higher proportion of MA in urine has been associated with a higher prevalence of urothelial carcinomas (Chen et al., 2005b; Chen et al., 2003b; Pu et al., 2007; Steinmaus et al., 2006a), skin cancer (Chen et al., 2003a;

Hsueh et al., 1997; Yu et al., 2000), cutaneous signs (Del Razo et al., 1997) and chromosomal aberrations (Maki-Paakkanen et al., 1998). A higher proportion of MA in urine indicates a lower methylating capacity and probably also a higher concentration of the highly toxic MA(III) in tissue leading to a higher retention of arsenic in the body.

As a large variation is seen in arsenic metabolism between individuals and population groups, genetic polymorphisms in genes coding for enzymes directly or indirectly involved in the methylation of arsenic have been suggested to be one of the main factors influencing the metabolism and thereby also the susceptibility to arsenic toxicity (Vahter, 2000). However, few studies have addressed this issue and additional studies are needed.

The methylation of arsenic is also likely to be dependent on the nutritional status, as several nutrients are involved in the strictly regulated one-carbon metabolism (**Fig. 1**). Availability of methyl groups is crucial for the maintenance of the methylation reactions, which are essential for numerous important cellular functions. Experimental studies have shown that rabbits with low intake of protein, choline or methionine decrease the ability to methylate arsenic (Vahter and Marafante, 1987). Some epidemiologic studies have shown associations between arsenic methylation ability and folate and B vitamins involved in the one-carbon metabolism (Gamble et al., 2005b; Steinmaus et al., 2005a). Furthermore, it has been shown that lower selenium intake could lead to reduced arsenic methylation and prolonged retention in the body (Chen et al., 1995; Hsueh et al., 2003).

1.4.3 Distribution

The valence state is of great importance for the cellular uptake of arsenic (Gebel, 2002). As(V) is a charged molecule at physiological pH 7.4 and is an analog to phosphate, thus, it probably enters the cell through non-specific anion transporters and other phosphate transporters (Gonzalez et al., 1995). As(III), however, is neutral and mimics glycerol and uses the aquaglyceroporin channels (Liu et al., 2004; Liu et al., 2002). After absorption in the intestine, both As(III) and As(V) are transported in the blood, mainly bound to sulfhydryl (SH) groups, to the liver. Only As(III) is taken up by the liver, where the main biotransformation occurs before inorganic arsenic and its methylated metabolites are distributed throughout the body. As(V) has been shown to be taken up by e.g. kidney or skeleton (Lerman et al., 1983; Lindgren et al., 1982).

Once inside the cell As(V) competes with phosphate and interferes with cellular metabolism, e.g. respiration, while some of it is reduced to As(III), which is the more reactive form. As(III) has a preference for SH-groups found in thiols such as glutathione (GSH) and thiol-containing proteins (Cui et al., 2006b; Lin et al., 2001; Vahter and Marafante, 1983; Zhou et al., 2006). Indeed, tissues high in SH-groups, e.g. keratin rich tissues such as hair, nails and skin showed a strong tendency to accumulate arsenic after intravenously administered radio labeled As(III) and As(V) in mice (Lindgren et al., 1982). Further, the highest levels of arsenic were found in liver, kidney and gallbladder. The results also revealed that the distribution of arsenic was dependent on the valence state of arsenic. After As(V) administration at fairly high concentrations the concentrations were lower in all organs than after As(III) administration, except in kidney and skeleton (Lindgren et al., 1982). This differences were reduced at low doses as As(V) is reduced to As(III) to a large extent. The higher concentrations seen in skeleton were probably due to that As(V) substitutes phosphate in hydroxyapatite, which is the main mineral component of bone.

The distribution of the different metabolites of inorganic arsenic in different tissues after As(V) administration has only recently been studied. It has been suggested that the distribution of inorganic arsenic and its metabolites differ both with tissue and animal species. In mice, the highest distribution of MA, DMA and iAs were seen in blood, bladder and kidney, respectively (Hughes et al., 2003). In hamsters, exposed to 5.0 mg As(III)/kg body weight, the liver contained mainly iAs, while the kidney and erythrocytes contained mostly MA (Naranmandura et al., 2007). In a study on mice administered an acute oral dose (0, 10 or 100 $\mu\text{mol As(V)/kg}$ body weight) it was demonstrated that blood, liver and kidney contain mainly iAs and MA and less DMA, while the opposite is true for urine (Kenyon et al., 2005).

Arsenic is able to cross the blood-brain barrier, but the concentrations in brain are considerably lower than in other tissues (Lindgren et al., 1982). Further, Concha and coworkers (Concha et al., 1998b) reported approximately equal levels of arsenic in cord blood as in maternal blood, showing that arsenic is readily transferred across the placenta barrier in humans.

1.4.4 Excretion

The major route of excretion is via the urine. When exposed to a single dose of inorganic arsenic the over all half-time is about four days and fits a three-component exponential function with 66% having a half time of about two days, 30% with a half time of about 10 days and 4% with a half time of about 38 days (Pomroy et al., 1980; Tam et al., 1979). However, this could well be different after chronic exposure with saturated binding sites.

Approximately 60% of the dose is excreted in urine and only about 6% in feces within a few days after oral administration (Pomroy et al., 1980). Experimental animals that have a more efficient methylation also have a more rapid excretion (Vahter and Marafante, 1983), which could explain the fact that arsenic is less toxic in most animals compared to humans. There are other routes of excretion for arsenic, e.g. hair, skin, nails, sweat and breast milk, however, the amounts excreted by those routes are insignificant in comparison with urine.

2 AIMS OF THE THESIS

The overall aim of the present thesis was to elucidate the mechanisms behind the marked inter-individual variation in the metabolism of inorganic arsenic in humans.

The more specific aims were:

- To assess the exposure to arsenic via drinking water and food in Central Europe and Bangladesh.
- To assess determinants influencing arsenic methylation in these populations, e.g. demographic factors, genetic polymorphisms, nutritional status and exposure level of arsenic.
- To provide an alternative to ICPMS as an arsenic-selective detector in settings where the cost of ICPMS is not justifiable.

3 MATERIALS AND METHODS

This chapter is a summary of the materials and methods used in this thesis. For further details in particularly on analytical procedures the reader is referred to the individual papers.

3.1 STUDY AREAS AND POPULATIONS

For the purpose of this thesis, data from two different case-control studies have been evaluated. The first case-control study concerns cancer risks in relation to low-dose arsenic exposure via drinking water in Central Europe: “ASHRAM – Arsenic Health Risk Assessment and Molecular Epidemiology” (**Paper II** and **III**). The second was a population based case-control study concerning the risk for skin lesions in relation to much higher arsenic exposure via drinking water in Bangladesh: “AsMat” – Arsenic in tube well water and health consequences in Matlab, Bangladesh (**Paper IV**). **Paper I** is a methodological study including samples from both low and high exposed individuals.

3.1.1 Hungary, Romania and Slovakia

Study area was defined as certain counties in Hungary (Bacs, Békés, Csongrad and Jasz-Nagykun-Szolnok), Romania (Bihar and Arad) and Slovakia (Banská Bystrica and Nitra) with known hotspots of arsenic in drinking water. New cases of skin, bladder and kidney cancer, along with hospital controls, were invited to participate in the study. Hospital controls, aged 30-79 years, were general surgery in-patients with appendicitis, abdominal hernias, duodenal ulcer or cholelithiasis and orthopaedic and traumatology patients with fractures.

3.1.2 Bangladesh

Study area was defined as Matlab Upazila, a rural area about 50 km south east of Dhaka, where the Meghna River joins the confluent streams of the Brahmaputra and Ganges rivers. About 60 percent of the tube wells in this area have arsenic concentrations above 50 µg/L, which is the Bangladeshi standard for drinking water, and about 70 percent are above the WHO guideline of 10 µg/L (Rahman et al., 2006b; Wahed et al., 2006). In Matlab, International centre for diarrhoeal disease research, Bangladesh (ICDDR,B) is running a comprehensive Health and Demographic Surveillance System (HDSS), including a population of 220,000. The Matlab area is divided into seven administrative areas (Block A-G). ICDDR,B has a hospital (in block

A) and four sub-centers (in blocks A-D) with health personnel in the area. Referents were randomly selected from the HDSS database with the criteria of being above four years of age, been living in the area for at least six months and drinking water from the area at least once a week.

3.2 ASSESSMENT OF ARSENIC EXPOSURE AND METABOLISM

A summary of the study design in the different papers is shown in **Table 2**. Arsenic in both urine and water were used for the assessment of arsenic exposure. In both studies spot urine samples were collected and stored in a freezer at -20°C or lower. Arsenic was determined in the urine samples either by performing speciation analysis of inorganic arsenic and its metabolites or by analyzing SumAs. In the European study water samples from self-reported sources were collected and stored at 4°C until analysis. In the Bangladeshi study all tube wells in the Matlab area ($N=13,286$) were screened for arsenic concentrations between January 2002 and August 2003 as part of the mitigation activities (Jakariya et al., 2007; Rahman et al., 2006b; Wahed et al., 2006). All tube wells found to contain more than $50\ \mu\text{g As/L}$ by field kit were immediately painted red and all containing less were painted green in order to guide the users to a well with less arsenic. A second water sample was collected and stored at -20°C until analysis. In both studies the participants were interviewed with regard to which drinking water sources they used at the time of the interview and in the past.

The fraction of DMA in relation to sum of arsenic metabolites in urine (%DMA) is correlated to the amount of DMA formed in the body and excreted in urine (% of the dose). Thus, on a group basis, a low %DMA in the urine indicates that the methylation efficiency is low, that the overall rate of excretion is low and that more arsenic (probably mainly as As(III) and MA(III)) is retained in the body (Vahter, 2002). This is also supported by a study of women and children in Argentina, where the ratio between arsenic in blood and urine increased with decreasing %DMA (Concha et al., 1998a), indicating that more arsenic was bound to blood and probably also other tissues at low methylation efficiency. Therefore, we have used, i.e. percent of inorganic arsenic (%iAs; As(III) + As(V); calculated as $\text{iAs}/\text{SumAs} \times 100$), percent of methylarsonate (%MA; calculated as $\text{MA}/\text{SumAs} \times 100$) and percent of dimethylarsinate (%DMA; calculated as $\text{DMA}/\text{SumAs} \times 100$) as a measure of the efficiency of the metabolism of inorganic arsenic.

Table 2. Summary of the study design used in the different papers.

Paper	Population	Samples			Analyses				
		Urine	Water	Blood	Speciation of As	SumAs	As in water	Geno-typing	Nutritional factors
I	Europe and Bangladesh	✓			✓	✓			
II	Europe	✓	✓		✓		✓		
III	Europe	✓		✓	✓			✓	Se, BMI
IV	Bangladesh	✓	✓	✓	✓	✓	✓		Ft, Zn, BMI, BSA, Crea

3.3 ANALYTICAL METHODS

3.3.1 Speciation of arsenic metabolites in urine

3.3.1.1 HPLC-HG-ICPMS

The speciation analyses of arsenic metabolites in the European study were performed at the Karl-Franzens University in Graz, Austria and in the Bangladeshi study at IMM, Karolinska Institutet, Sweden. For speciation analyses of arsenic metabolites in urine an ICPMS (HP 4500, Agilent 7500cs or Agilent 7500ce, Agilent Technologies, Waldbronn, Germany) equipped with an integrated sample introduction system (ISIS) and an HG accessory together with an Agilent 1100 chromatographic system equipped with solvent degasser, autosampler, and a thermostatted column were used. For the separation of As(III), DMA, MA, and As(V) a Hamilton PRP-X100 anion-exchange column (4.6 mm x 250 mm) was used.

3.3.1.2 HPLC-HG-AFS

In an attempt to find an alternative to HPLC-HG-ICPMS for speciation analysis of arsenic metabolites in urine a HG-AFS was evaluated (PSA 10.055 Millennium Excalibur system; PS Analytical Ltd, Orpington, Kent, UK) as detection system for the arsenic compounds.

3.3.2 Determination of arsenic in urine and water with HG-AAS

In the Bangladeshi study determination of SumAs in urine was performed with direct HG-AAS after addition of HCl at IMM, Karolinska Institutet, Sweden. This method and equipment are described in more detail elsewhere (Norin and Vahter, 1981; Vahter et al., 1986). For determination of total arsenic concentration in drinking water HG-AAS after addition of HCl and KI combined with heating was used. In the European study these analyses were performed at the Environmental Health Centre in Cluj-Napoca, Romania with some duplicate analyses at the Karl-Franzens University in Graz, Austria and in the Bangladeshi study these analyses were performed at ICDDR,B in Dhaka with some duplicate analyses at IMM, Karolinska Institutet, Sweden.

3.3.3 Determination of nutritional markers

3.3.3.1 Hungary, Romania and Slovakia

Whole blood samples were collected and kept frozen at -20°C until selenium analysis. Selenium in whole blood was analyzed at a commercial laboratory (Analytica AB, Luleå, Sweden) with inductively coupled plasma sector field mass spectrometry (ICPSFMS; ELEMENT, ThermoElectron, Finnigan MAT, Bremen, Germany) monitoring m/z 78 in high resolution mode ($\Delta m/m=11\ 000$).

3.3.3.2 Bangladesh

Both ferritin and zinc were analyzed at the University of California, Davis, USA. Ferritin was measured in plasma samples using radioimmunoassay (Diagnostic Products, San Diego, CA, USA). Zinc was assessed in plasma samples by AAS (Clegg et al., 1981). Urinary creatinine was determined at Karolinska University Hospital, Huddinge, Sweden, using the Jaffe method (Hare, 1950).

3.3.4 Adjustment for dilution in urine samples

As previously pointed out, spot urine samples needs to be adjusted for the variation in dilution when comparing concentrations between individuals and populations. Either, the arsenic concentration in urine is related to the urinary creatinine excretion or adjusted to the mean specific gravity of the urine in the studied population. As creatinine excretion is considerably more influenced by body size, age and gender than the specific gravity (Nermell et al., 2007), all concentrations measured in urine were adjusted to the average specific gravity in the respective population. The specific gravity was measured with a refractometer as the angle of light refraction between air

and urine sample (Leica TS 400 Refractometer, Leica Microsystems Inc., Buffalo, NY, USA and Uricon-Ne, ATAGO Co. Ltd, Tokyo, Japan).

3.3.5 Genotyping

The genotyping in the European study was performed at the German Cancer Research Center in Heidelberg, Germany. DNA was isolated from whole blood samples, stored at -80°C, using Qiagen mini-preparation kits and genotyped for single nucleotide polymorphisms (SNPs) in the methylene tetrahydrofolate reductase (MTHFR; Unigene Accession number Hs.214142, (NCBI, 2007)), glutathione-S-transferase omega 1 (GSTO1; Hs.190028) and arsenic (III) methyltransferase (AS3MT; Hs.34492) genes. Genotyping was performed by the 5' nuclease allelic discrimination assay (TaqMan) in 96-well format as described previously (Thirumaran et al., 2006). Four percent of genotyping results from allelic discrimination assays were randomly verified by direct DNA sequencing. The sequencing reactions were performed using BigDyeR Terminator Cycle sequencing kit (Applied Biosystems) in a 10 ml volume containing PCR product pre-treated with ExoSapIT (Amersham Biosciences, Uppsala, Sweden) and a sequencing primer.

3.4 ETHICAL CONSIDERATIONS

Informed consent was obtained from all participants. The European study (**paper II** and **III**) was approved by each separate hospital's ethics committee. The Bangladeshi study (**paper IV**) was approved by both the ICDDR,B Ethical Review Committee as well as the Ethics Committee at the Karolinska Institutet in Stockholm. **Paper I** is a method comparison study and therefore, no ethical approval is needed.

3.5 STATISTICAL ANALYSES

Statistica 7.1 for Windows (StatSoft. Inc., Tulsa, OK, USA) and SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA) were used to perform the statistical analyses. Spearman correlation test (r_s) was used when testing for univariate associations between continuous variables. Non-parametric tests, Mann-Whitney U-test and Kruskal Wallis test, were used when testing for univariate differences between groups. One-way analysis of variance (ANOVA) was used when testing for univariate differences between genotypes. Pearson Chi-square was used when testing for differences between categorical variables. When using linear regression, the variables were transformed, if required, to meet the requirement of equal variance and

normal distribution of residuals. Tests for collinearity (tolerance and variance inflation factors) were performed and when the collinearity was too high the independent variable giving the highest R square of the model was chosen. Possible interactions in the models were explored. To further explore interactions analysis of covariance (ANCOVA) was used. P-values <0.05 were used for statistical significance, except for interactions where a p-value <0.10 was used.

4 RESULTS AND DISCUSSION

The main findings, including some unpublished data, and a general discussion of these are presented in this chapter. For further details the reader is referred to the individual papers.

4.1 ANALYTICAL METHODS FOR DETERMINATION OF ARSENIC METABOLITES

Due to the extent of arsenic contaminated drinking water all over the world, especially in low-income countries (WHO, 2001), there is a need for exposure monitoring and evaluations of mitigation activities in these countries. Therefore, fast, simple and low cost analytical methods for these purposes are needed. We have further developed a previously described HPLC-HG-AFS method (**Fig 2.**) for determination of inorganic arsenic and its metabolites in urine (Gomez-Ariza et al., 1998). We compared the obtained results with those of two other commonly used analytical methods (**Paper I**). HPLC-HG-ICPMS is a well-documented method; however, it is expensive for many laboratories. The ICPMS instrumentation cost about 10 times more and it consumes about 70 times more argon gas compared to AFS.

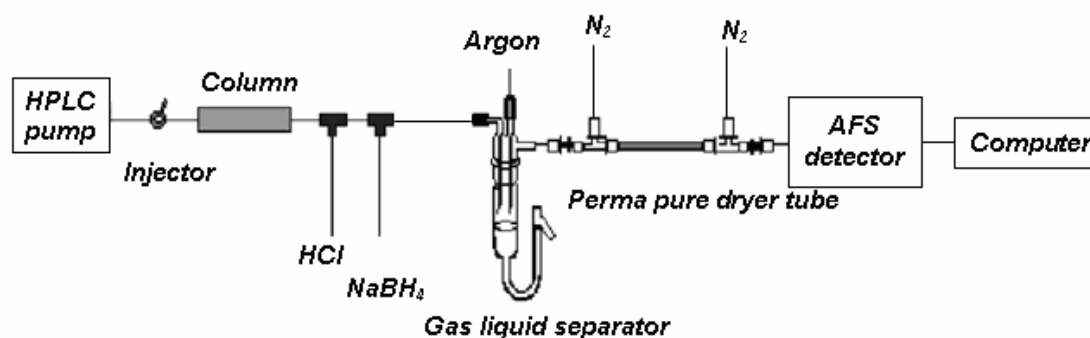


Figure 2. Schematic diagram of the instrumental set-up of the HPLC-HG-AFS

For evaluation of the HPLC-HG-AFS method both a between-laboratory and a within-laboratory comparison were performed. In the between-laboratory comparison, urine samples were analyzed with HPLC-HG-AFS at IMM, Karolinska Institutet, Sweden and with HPLC-HG-ICPMS at Karl-Franzens-University, Graz, Austria. In the within-

laboratory comparison, urine samples were analyzed with HPLC-HG-AFS, HPLC-HG-ICPMS and HG-AAS at IMM, Karolinska Institutet, Sweden. The results of the comparisons are showed in **Table 3**. The between-laboratory comparison showed a reasonable agreement between the two methods and the different metabolites. The within-laboratory comparison showed better agreement for the different metabolites when comparing HPLC-HG-AFS and HPLC-HG-ICPMS than in the between-laboratory comparison. HPLC-HG-AFS was thus found to be a good alternative to HPLC-HG-ICPMS for speciation analysis of arsenic in urine in these highly exposed areas where the requirements of low detection limits are not needed.

Table 3. Regression coefficients and equations of the between- and within-laboratory comparisons for the different arsenic species.

		R ²	Slope	Intercept
Between-laboratory comparison (N=86)				
HPLC-HG-ICPMS vs. HPLC-HG-AFS	iAs ^a	0.91	0.83	0.53
	MA	0.92	0.84	0.56
	DMA	0.90	1.1	-0.84
	SumAs	0.92	1.0	0.22
Within-laboratory comparison (N=89)				
HPLC-HG-ICPMS vs. HPLC-HG-AFS	iAs ^a	0.78	1.4	1.5
	MA	0.85	1.1	0.71
	DMA	0.96	0.95	1.2
	SumAs	0.95	1.1	1.5
HG-AAS vs. HPLC-HG-AFS	SumAs	0.95	1.1	-2.8
HPLC-HG-ICPMS vs. HG-AAS	SumAs	0.97	0.98	5.1

^a iAs is the sum of As(III) and As(V)

The between- and within-laboratory comparisons show that the quality of the analytical measurements was good. We used between- and within-laboratory comparisons to certify the quality of the different arsenic analyses because there are no certified reference materials for the toxicologically relevant arsenic species in urine, i.e. As(III), As(V), MA and DMA. The only commercially available reference material for arsenic

species in urine is NIES CRM no. 18 (Yoshinaga et al., 2000). Unfortunately, the only certified arsenic species are AB and DMA.

4.2 ARSENIC EXPOSURE

The arsenic exposure was assessed in both Central Europe (**Paper II**), including Hungary, Romania and Slovakia, and in Bangladesh using arsenic concentrations in urine and drinking water (**Paper IV; Table 4**). The exposure was significantly higher in the Bangladeshi population with approximately 10 times higher arsenic concentrations in both urine and drinking water than in the European population. Within the European study, Hungarians had significantly higher concentrations in both drinking water and urine than Romanians and Slovaks. A large variation in exposure was seen in both populations.

Table 4. Arsenic concentrations in urine and water (**Paper II** and **IV**).

		SumAs ($\mu\text{g/L}$)	W-As ($\mu\text{g/L}$)
Hungary, Romania and Slovakia	N	520	537
	Min	0.30	0
	5th Perc	1.0	0.34
	Median	6.0	1.3
	95th Perc	50	39
	Max	140	95
Bangladesh	N	1,571	1,571
	Min	0.47	0
	5th Perc	20	0
	Median	77	14
	95th Perc	453	461
	Max	1,994	3,644

The main source of arsenic exposure was found to be drinking water in both study areas. The association between arsenic in drinking water and urine had a coefficient of determination (R^2) of 0.46 and 0.41 in the European study and in the Bangladeshi study, respectively (**Fig. 3A** and **B**, respectively). The fact that only about 40% of the concentration in water explains the concentrations found in urine indicates that the

arsenic concentration in water is not an optimal exposure marker. However, when evaluating the risk for health effects with latency periods of at least 5-10 years, which is the case for arsenic, a measure of long-term exposure is needed. In these studies arsenic concentrations in water are the only available marker of exposure, as urine samples are not feasible to collect from a large number of individuals during a long time period.

A large variation was seen in the urine concentrations at one and the same water concentration, which can be seen in both **Fig. 3A** and **B**. In Europe, this variation is probably due to consumption of other waters and beverages. However, in Bangladesh where other beverages are not extensively used the probable reason is consumption of water from other water sources than the self reported drinking water source, as wells could vary considerably in concentration even though they are located close to each other (Jakariya et al., 2005).

The intercepts in the regression equations between arsenic in water and urine (where the water concentrations are equal to zero) in both the European and Bangladeshi study were calculated in an attempt to assess the background exposure from food. The background exposure was calculated to be around 2.5 $\mu\text{g/L}$ in urine in the European study and around 45 $\mu\text{g/L}$ in the Bangladeshi study indicating that not only the arsenic exposure from water, but also from food were significantly larger in Bangladesh than in Europe. In Europe, a higher proportion of DMA was found in urine at concentrations below 2.5 $\mu\text{g/L}$ in urine, indicating that the arsenic exposure from food was mainly in the form of DMA or arsenosugars, which partly are metabolized to DMA in the body (Buchet et al., 1994; Francesconi et al., 2002; Heinrich-Ramm et al., 2002; Le et al., 1994; Ma and Le, 1998; Raml et al., 2005; Schmeisser et al., 2004). In Bangladesh, the diet mainly consists of rice (Sudo et al., 2004), which has been shown to contain considerable amounts of inorganic arsenic (Williams et al., 2005). The main part of the background exposure was probably in the form of inorganic arsenic as the proportion of DMA was not that different below 45 $\mu\text{g/L}$ in urine. It is important to consider which chemical form of arsenic people are exposed to when performing exposure assessments as inorganic arsenic in general is more toxic than the organic species (Hughes and Kenyon, 1998).

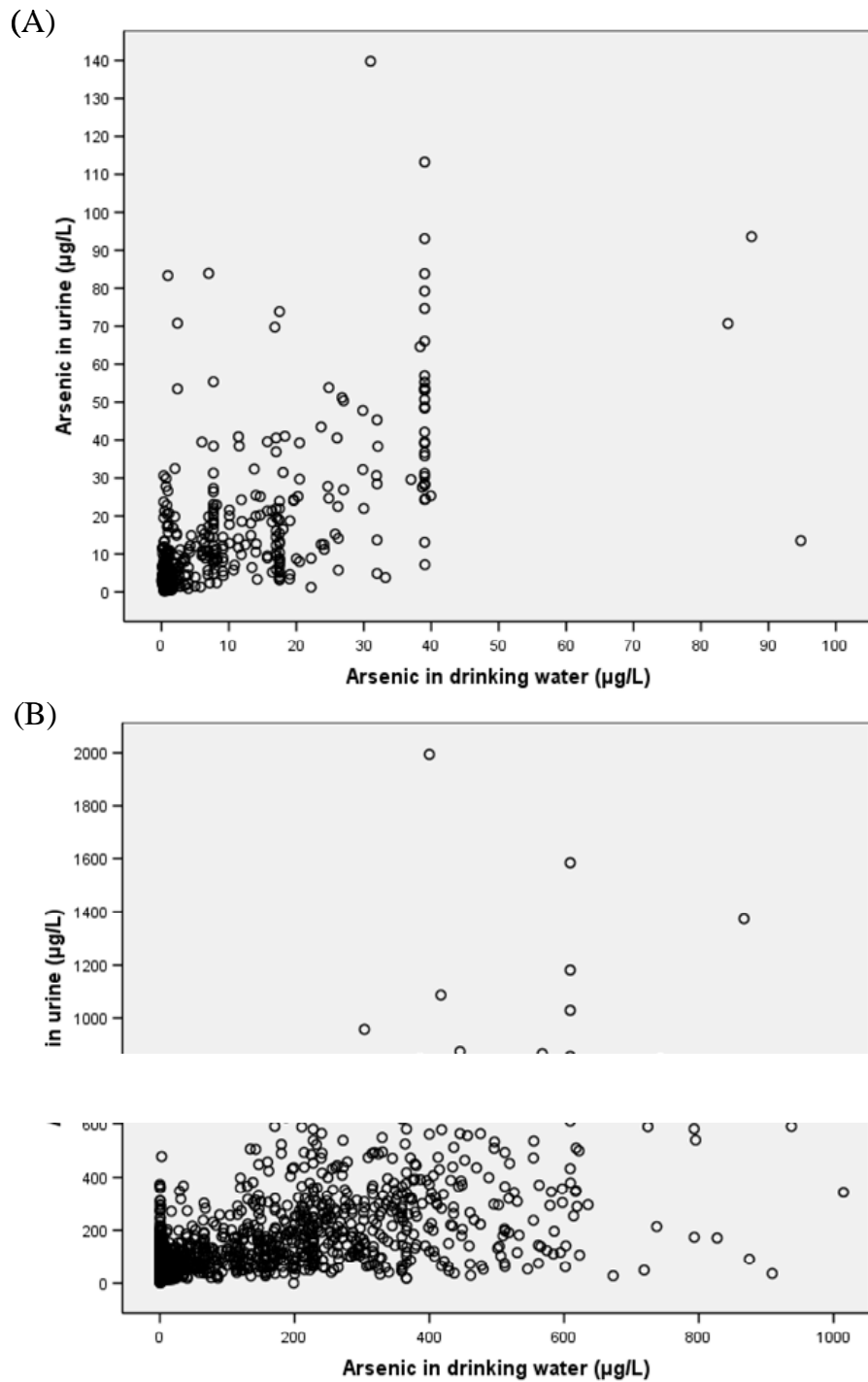


Figure 3. Association between urinary arsenic concentrations and arsenic concentrations in drinking water in Hungary, Romania and Slovakia (A; **Paper II**) and Bangladesh (B; **Paper IV**). One individual from Bangladesh with 3644 $\mu\text{g/L}$ in drinking water was excluded.

4.3 ARSENIC METABOLISM

We could confirm previous findings that there is a large inter-individual variation in the distribution of inorganic arsenic and its metabolites in urine. The relative standard deviations were 55% and 44% in Europe and Bangladesh, respectively (**Fig. 4**). There was also a large difference in the proportion of MA in urine between the two populations (mean: 16 and 12% in Europe and Bangladesh, respectively). We were not able to confirm previous studies showing substantial amounts of MA(III) and DMA(III) in urine (Aposhian et al., 2000; Le et al., 2000; Mandal et al., 2001; Valenzuela et al., 2005). The probable reason is that the high reactivity of these metabolites render them to bind in tissue and the rate of oxidation to the pentavalent forms are very high (Nakayama et al., 2006; Vahter, 2002).

As previously discussed, several studies indicate an association between a higher %MA and a higher prevalence of arsenic induced toxic effects (Chen et al., 2003a; Chen et al., 2005b; Chen et al., 2003b; Del Razo et al., 1997; Hsueh et al., 1997; Maki-Paakkanen et al., 1998; Pu et al., 2007; Steinmaus et al., 2006a; Yu et al., 2000). Therefore, it is of great importance to study different factors influencing the metabolism of inorganic arsenic in order to be able to identify potential subgroups with a higher risk for toxic effects caused by arsenic exposure. Several factors have been suggested to influence the metabolism of inorganic arsenic, e.g. age, gender, genetic polymorphisms in genes coding for enzymes involved in arsenic metabolism, exposure level of arsenic, nutrition, smoking, alcohol use, diseases etc (Vahter, 2002). This chapter will focus on several of the above mentioned factors (**Table 5**). The combination of these two contrasting populations gives an excellent opportunity to study the importance of these factors on arsenic metabolism and to increase the understanding of the underlying mechanisms.

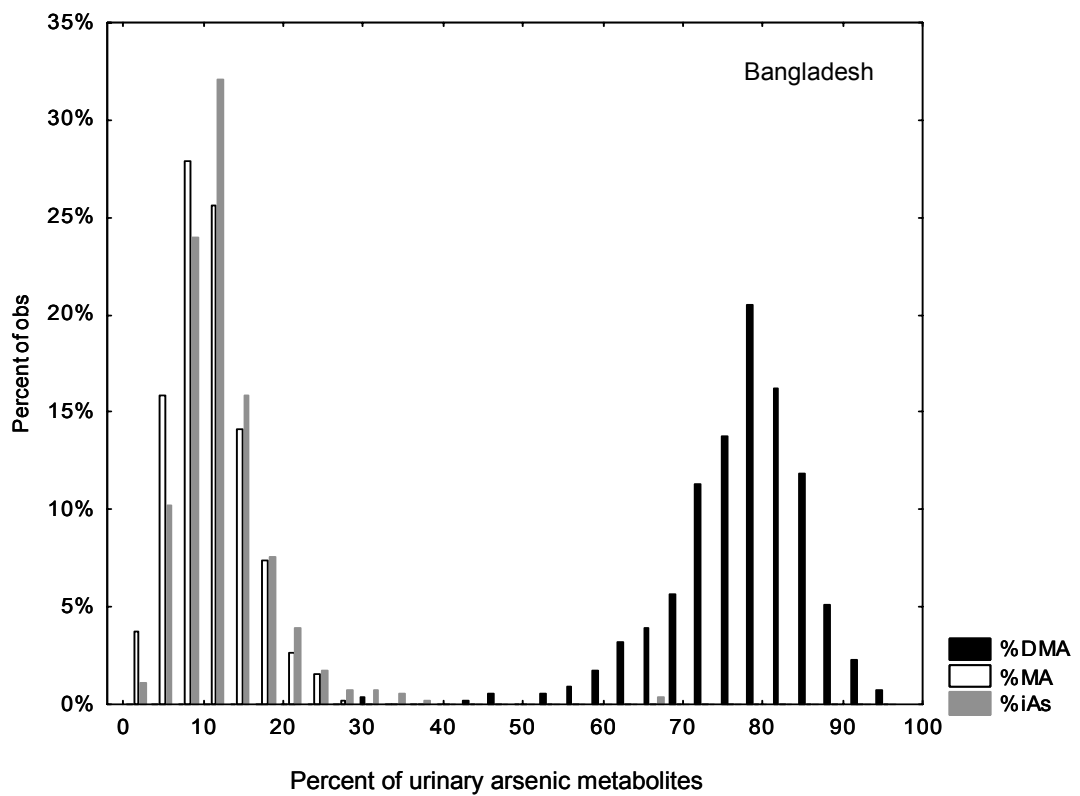
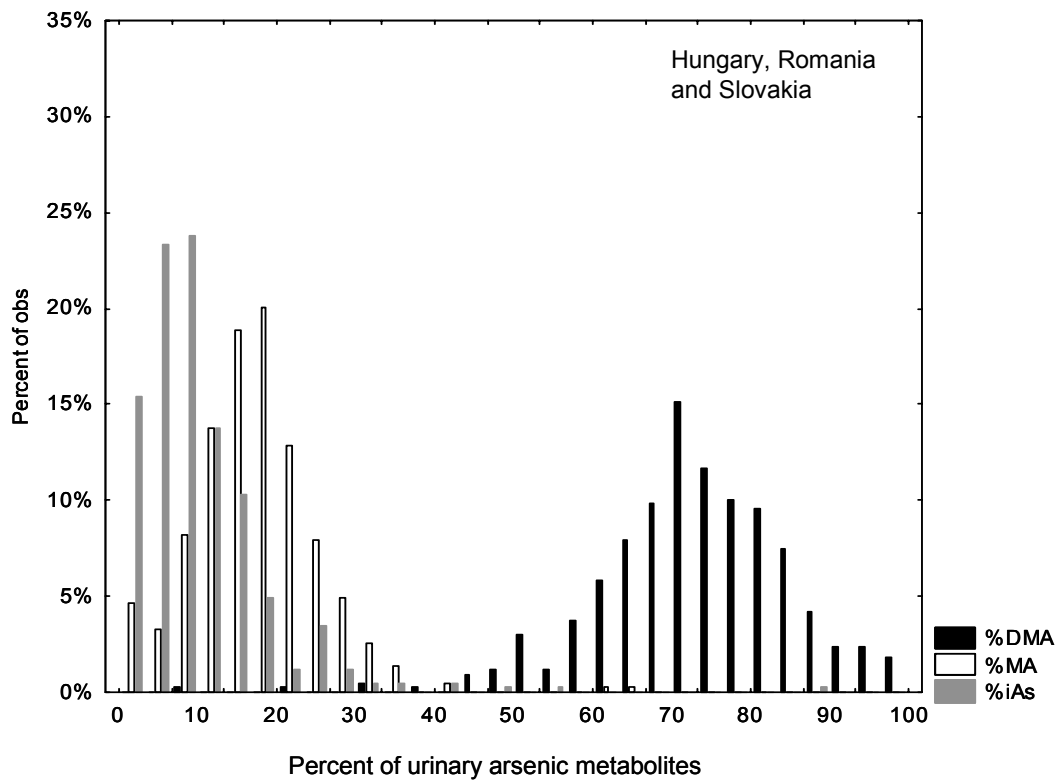


Figure 4. Distribution of the proportions of inorganic arsenic (%iAs), methylarsonate (%MA) and dimethylarsinate (%DMA) in urine in the two populations studied. Individuals with SumAs less than 2 µg/L have been excluded in the European study.

Table 5. Factors possibly influencing arsenic metabolism elucidated in the different papers.

		Hungary, Romania and Slovakia (Paper III)	Bangladesh (Paper IV)
Demographic factors	Gender	✓	✓
	Age	✓	✓
	Tobacco use	✓	✓
	Socio-economic status		✓
Genetic polymorphisms	AS3MT	✓	
	MTHFR	✓	
	GSTO1	✓	
Nutrition	Selenium	✓	
	Ferritin		✓
	Zinc		✓
	BMI ^a	✓	✓
Arsenic exposure	Exposure level of arsenic	✓	✓

^a Body mass index

4.3.1 Variation by demographic factors

As shown in **Table 5**, gender, age and tobacco use were all evaluated in both studies. In accordance with previous studies (Gamble et al., 2005b; Hopenhayn-Rich et al., 1996b; Loffredo et al., 2003), men were less efficient in methylating arsenic to DMA compared to women in both studies, with both higher %iAs and %MA. Age was an important factor in the Bangladeshi study, but not in the European study. This was probably due to the narrow age span in the European study (age range: 28-83 years) compared to the Bangladeshi study (age range: 6-88 years). Children and adolescents (only included in the Bangladeshi study) were more efficient in methylating arsenic to DMA than adults, and had lower %MA and %iAs than the adults. This could perhaps be due to an increased overall methylation in the body during the period of major growth. Indeed, the expression of genes coding for methyltransferases involved in DNA methylation decreases significantly with age in humans (Zhang et al., 2002). Furthermore, other inhibitory factors such as smoking, alcohol consumption and exposure to other environmental pollutants all increase with age and could also contribute to the decreasing methylating capacity with age.

Interestingly, an interaction between gender and age was found for %MA in both studies showing that only women between 20 and 60 years of age had lower %MA than men of the same age. This difference was not seen among boys and girls in the Bangladeshi study, nor was it seen in people above 60 years of age in either of the Bangladeshi or the European studies (**Fig. 5**). These results indicate an effect of sex hormones on arsenic methylation. Estradiol and testosterone, two of the most common sex hormones in the body, have been shown to influence other methylation reactions, e.g. DNA methylation (Anway et al., 2006; Kumar and Thakur, 2004).

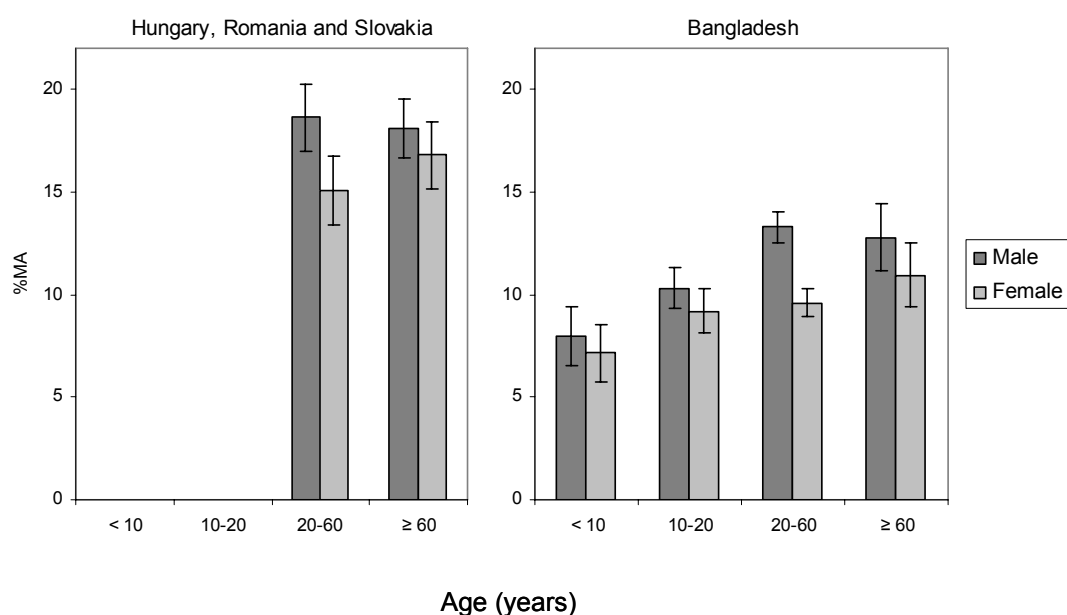


Figure 5. Mean and corresponding confidence intervals of the proportion of methylarsonate (%MA) by different ages in men and women (**Paper III** and **IV**).

We were able to confirm that tobacco smoking negatively influenced arsenic methylation, but only among adult men in the Bangladeshi study, not in the European study. We further investigated if the effect of tobacco smoking on arsenic methylation was influenced by arsenic exposure. This was done by performing ANOVA among adult men in the Bangladeshi study with %MA as the dependent variable and smoking status (no=non-smokers and yes=smoking cigarettes or bidi) and urinary arsenic concentrations divided into tertiles as fixed factors (**Fig. 6**). The influence of smoking was not seen in the lowest tertile and could thereby explain why we were not able to see this association in the European study. Furthermore, %MA increased only from 10% to 12% in non-smokers from the lowest tertile to the highest tertile of SumAs, compared to smokers in which %MA increased from 11% to 16%, suggesting an

additive effect between tobacco smoking and arsenic exposure on arsenic methylation. This is also supported by the literature where a significant association between smoking and arsenic methylation is almost exclusively found in populations with high arsenic exposure and not in populations with low exposure (Hopenhayn-Rich et al., 1996b; Pu et al., 2007; Steinmaus et al., 2006a; Steinmaus et al., 2005a). This might also explain the synergistic interaction found between arsenic exposure and smoking when evaluating the risk of lung cancer (Hertz-Picciotto et al., 1992).

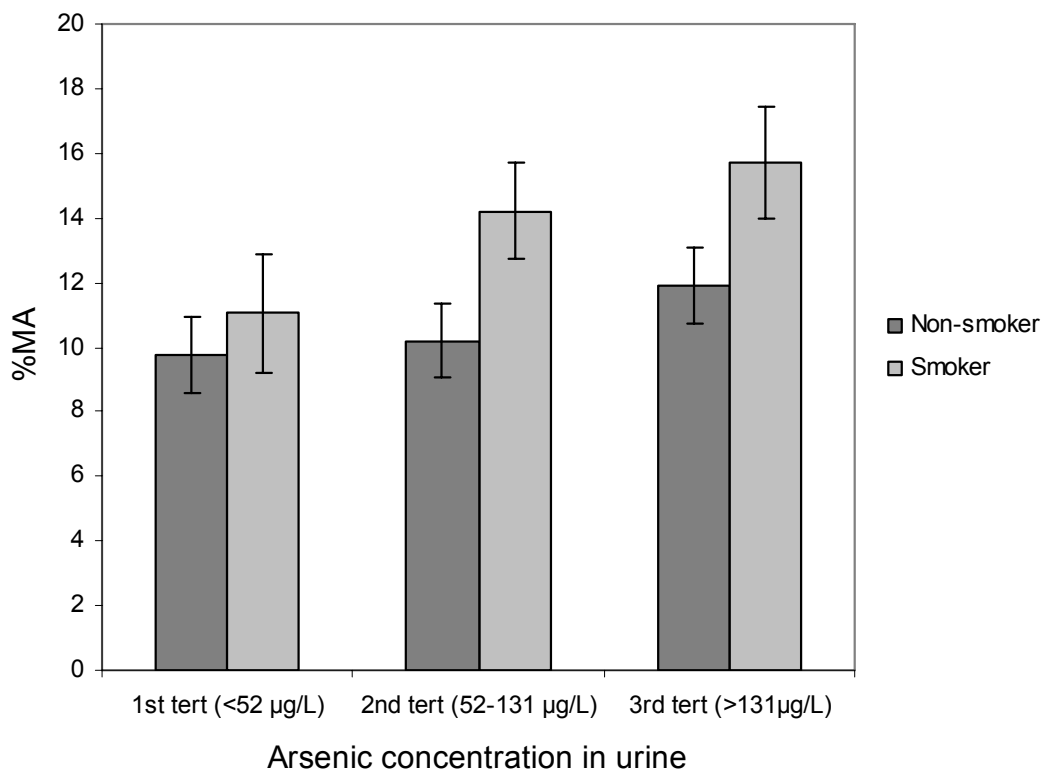


Figure 6. Mean and corresponding confidence intervals of the proportion of methylarsonate (%MA) by tertiles of urinary arsenic concentration in male Bangladeshi smokers and non-smokers (unpublished data).

4.3.2 Variation by genetic polymorphisms

Only five studies, with somewhat contradictory results regarding the influence of SNPs on arsenic methylation were found in the literature, most of which include a limited number of participants. Polymorphisms have been studied in genes coding for enzymes directly involved in arsenic metabolism, e.g. AS3MT and GSTs, and enzymes involved in one-carbon metabolism, e.g. MTHFR and 5-methyltetrahydrofolate homocysteine methyltransferase (MTR). In the present study (**Paper III**) we elucidated the effects of SNPs in the AS3MT, MTHFR and GSTO1 genes on arsenic methylation.

We found that the M287T (C>T) polymorphism in the AS3MT gene was associated with decreased %DMA and increased %MA. Interestingly, this polymorphism did not influence arsenic methylation as much in women as it did in men. However, Meza and coworkers (Meza et al., 2005) found an association between other SNPs in the AS3MT gene in a Mexican population and increased DMA/MA ratio, indicating a more efficient methylation. This is confirmed in a very recent study performed on indigenous Argentinean women, which showed an association between SNPs in the AS3MT gene and increased methylation efficiency (Schl awicke Engstr om et al., 2007). However, we were not able to find the same SNPs in the present study. This could be the reason why people with indigenous origin have been shown to have a very unique metabolism compared to other populations (Vahter et al., 1995a). Thus, different SNPs in the same gene could have opposing effects.

Polymorphisms in the MTHFR gene were also associated with higher %MA and lower %DMA in the present study. This is in agreement with other studies, both performed in Argentina (Schl awicke Engstr om et al., 2007; Steinmaus et al., 2007). Schl awicke Engstr om and coworkers also studied polymorphisms in the 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) gene, but did not find an association with arsenic methylation. Interestingly, in the present study we found that a mutation on only one allele was needed to alter the arsenic methylation in men, while women needed mutations on both alleles. Further, we found that individuals above 60 years of age were more affected by polymorphisms in the MTHFR gene. One explanation could be that older individuals often have lower levels of vitamin B₁₂ (Martin, 2006), which in combination with polymorphisms in MTHFR cause higher levels of homocysteine, indicating a reduced overall methylation capacity in the body (Holm et al., 2007).

An association between polymorphisms in GSTO1 and arsenic methylation was not found, in contrast to a previous study (Marnell et al., 2003). Marnell and coworkers found that two individuals with an uncommon genotype of GSTO1 had a much higher proportion of inorganic arsenic and less methylated metabolites in urine than the other participants. In accordance with the present study Meza and coworkers (Meza et al., 2005) were also not able to confirm an association between SNPs in GSTO1 gene and arsenic methylation. Chiou and coworkers (Chiou et al., 1997) elucidated the effect of SNPs in glutathione-S-transferase mu 1 (GSTM1) and glutathione-S-transferase theta 1

(GSTT1) on arsenic methylation in a Taiwanese population. They found that the wild type of a GSTM1 polymorphism was associated with higher %iAs. Schläwicke Engström and coworkers also studied SNPs in GSTM1 and GSTT1 and found that SNPs in GSTM1 were associated with lower %MA and GSTT1 with higher %MA. Steinmaus and coworkers (Steinmaus et al., 2007) were not able to confirm the association between GSTM1 or GSTT1 and arsenic methylation efficiency. However, the exposure level was much lower and over 80% of the participants were men.

4.3.3 Variation by nutritional status

Malnutrition has been shown to influence the susceptibility to arsenic induced health effects, probably due to low anti-oxidative defense, and also indirectly by influencing arsenic methylation (Gamble et al., 2005b; Milton et al., 2004; Mitra et al., 2004; Steinmaus et al., 2005a). Different markers of nutritional status were used in the different studies (**Table 5**). The results from the present study (**Paper III** and **IV**) showed that nutrition did not influence the metabolism of inorganic arsenic as much as previously believed.

Body mass index (BMI) and selenium in whole blood were evaluated in the European study (**Paper III**). However, as 65% of the participants were either overweight or obese, BMI was not a good measure of nutritional status, but rather a measure of the amount of adipose tissue. Still, we did find a positive association between BMI and arsenic methylation, indicating that increased adipose tissue increases the methylation efficiency. The difference was largest between normal weight men and overweight and obese women. These results may support our previous hypothesis that sex hormones influence arsenic methylation, as estrogen is produced in adipose tissue (Nelson and Bulun, 2001). Further, sex hormone binding globulin (SHBG) is negatively associated with body weight, leading to a release of free estrogen and progesterone from SHBG with increasing BMI (Pugeat et al., 1995). Selenium was found to be negatively associated with arsenic methylation, in contrast to previous studies (Hsueh et al., 2003; Jay Christian et al., 2006). However, during further evaluation it was revealed that Romanians and Slovaks had lower selenium concentrations, but higher %DMA, resulting in the negative association. Therefore, it is most likely not a causal association, but rather an effect of e.g. different food habits in the different countries.

In the Bangladeshi study, BMI together with ferritin and zinc in plasma were used as nutritional markers (**Paper IV**). In contrast to previous studies (Gamble et al., 2005b; Steinmaus et al., 2005a), none of the three markers were significantly associated with arsenic methylation after multivariate adjustment of gender, age and exposure level. The discrepancy between the present study and previous studies (Gamble et al., 2005a; Steinmaus et al., 2005a) in finding an association between nutritional status and arsenic methylation could be due to that we were not able to measure folate and B-vitamins that are directly involved in the one-carbon metabolism. However, in this population, where over 40% of the participants were underweight, BMI is probably a relatively good marker for nutritional status. BMI was also highly correlated with ferritin ($p < 0.001$), but not with zinc ($p = 0.7$). We conclude, based on the efficient methylation in the Bangladeshi population in combination with the high prevalence of malnutrition and the lack of association between arsenic methylation and BMI, ferritin or zinc, that nutritional status has a small impact on arsenic methylation. This is probably due to the large flexibility of the numerous essential methylation reactions in the body (Brosnan et al., 2004; Holm et al., 2007; Niculescu and Zeisel, 2002; Ueland et al., 2005).

4.3.4 Variation by exposure level of arsenic

The exposure level of arsenic has previously been shown to influence arsenic metabolism (Del Razo et al., 1997; Hopenhayn-Rich et al., 1996a; Hopenhayn-Rich et al., 1996b; Kurttio et al., 1998; Tseng et al., 2005), however, with some exceptions (Hsueh et al., 2003; Steinmaus et al., 2005b). At elevated arsenic exposure in the Bangladeshi study, the urinary arsenic concentration, reflecting the current exposure, was found to be one of the strongest influencing factors on arsenic metabolism and the association was negative (**Fig. 7, Paper IV**). However, at low exposure levels this effect was not seen (**Paper III**).

Probably the increasing methylation efficiency at higher exposure levels are inhibition of methyltransferases involved in arsenic methylation, as shown *in vitro* (Drobna et al., 2005). Most likely, this is not due to shortage of SAM (Csanaky et al., 2003). Arsenic has been shown to inhibit a variety of enzymes, including methyltransferases involved in DNA methylation (Chen et al., 2004; Cui et al., 2006a; Cui et al., 2006b; Reichard et al., 2007; Zhou et al., 2006). In agreement with *in vitro* studies (De Kimpe et al., 1999b; Drobna et al., 2005), it seems to be particularly the second methylation step that is most sensitive to excess arsenic exposure, with decreased DMA, increased MA and

fairly constant iAs. Decreased methylation of arsenic has been shown to lead to increased retention times in the body (Vahter, 1999b). This is supported by the finding that the ratio between urinary arsenic and arsenic concentration in water decreased with increasing exposure (**Fig. 2 in Paper IV**).

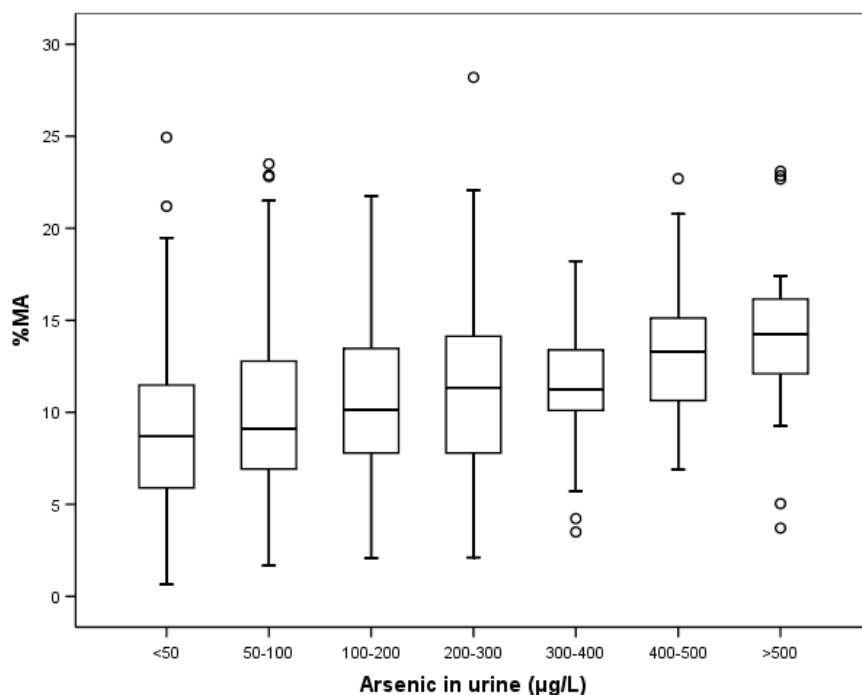


Figure 7. Relationship between %MA and urinary arsenic divided into categories in the Bangladeshi population (**Paper IV**).

4.3.5 Variation between population groups

As previously mentioned there are large variations in arsenic metabolism between human population groups exposed to inorganic arsenic. This study was not an exception. In spite of the much higher exposure in Bangladesh, they had lower %MA in urine than the European population (mean: 16 and 12% in Europe and Bangladesh, respectively; **Fig. 8**). The Europeans were found to be similar to most populations in regard to %MA. However, they had lower %iAs than the other populations, which most likely is due to the low exposure to iAs and the contribution of DMA from food. The people in Bangladesh had an efficient methylation to DMA, with only small amounts of MA and iAs, compared to most other populations, with the exception of the indigenous people in Argentina with their completely different metabolite pattern (Vahter et al., 1995a). The metabolite pattern of arsenic in urine in the Bangladeshi population was

also quite similar to that previously reported from Bangladesh (Chowdhury et al., 2003; Gamble et al., 2006; Gamble et al., 2005b).

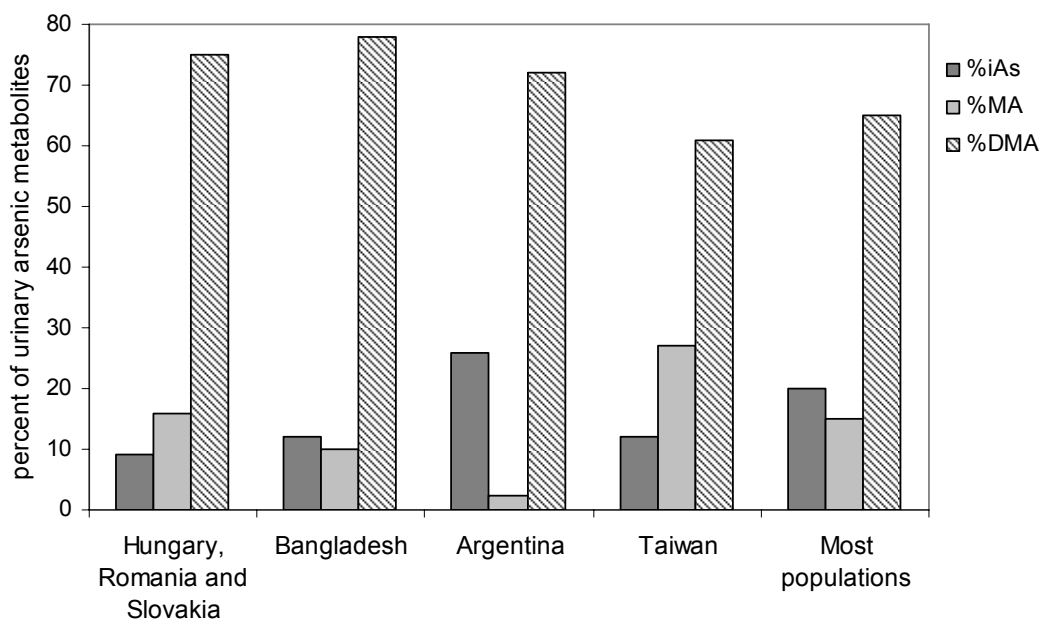


Figure 8. Proportions of arsenic metabolites, iAs, MA and DMA, in urine in different populations: people in certain counties in Hungary, Romania and Slovakia (**Paper III**), people in Matlab, Bangladesh (**Paper IV**), indigenous people in the Andes in Argentina (Vahter et al., 1995a), Taiwanese in south-west Taiwan (Chiou et al., 1997) and the average in most populations studied (Hopenhayn-Rich et al., 1993; Vahter, 2002).

The efficient methylation of inorganic arsenic by the people in Bangladesh was in direct contrast to our expectations, due to the high prevalence of elevated arsenic exposure and malnutrition. These findings further support that nutrition is not as important as previously hypothesized and that genetic polymorphisms probably are the main determinants for arsenic metabolism. This is also supported by the efficient methylation of the rather isolated indigenous population in the Andes, who have been exposed to arsenic from generation to generation for thousands of years (Vahter, 2002). Interestingly, the prevalence of skin lesions seems to be rather low in the Andean population (Schl awicke Engstr om et al., 2007). It could be speculated that this is the result of a natural selection towards having an efficient methylation. That an efficient methylation of arsenic has a protective effect against arsenic induced toxicity is further supported by several studies as discussed previously (Chen et al., 2003a; Chen et al., 2005b; Chen et al., 2003b; Del Razo et al., 1997; Hsueh et al., 1997; Maki-Paakkanen et al., 1998; Pu et al., 2007; Steinmaus et al., 2006a; Yu et al., 2000). However, this is

not applicable to people in Bangladesh, who have been exposed to arsenic for only 20-30 years. A possible explanation could be that, the high prevalence of malnutrition in Bangladesh through history has resulted in an adaptation to an efficient methylation in general, as methylation reactions are essential in the body and with the assumption that arsenic could be methylated by non-arsenic specific methyltransferases.

Other reasons for the different metabolite patterns in Europe and Bangladesh could be that the participants in the European study were older, which was shown to influence the metabolism negatively. However, the impact of age, especially among adults, was so small that it could hardly explain the whole difference in higher %MA. Other differences between Europe and Bangladesh could influence the metabolism of inorganic arsenic, including more prevalent alcohol consumption and more women smoking tobacco. Europe is also far more industrialized than Bangladesh. All these factors are probably leading to inhibition of the methylation (De Kimpe et al., 1999a; Vahter, 2002). This is also supported by the larger inter-individual variation in Europe compared to Bangladesh (**Fig. 4**).

Although we have studied a large number of factors in the present study, they only explain a small part of the variation, on an estimate only 20%. Obviously, there are several other factors influencing arsenic metabolism. It is unlikely that AS3MT is the only methyltransferase that catalyzes the methylation reactions, given that there are around one hundred different methyltransferases identified in the human body (Martin and McMillan, 2002). Furthermore, because methylation reactions in the body are so important for so many biological functions, the different pathways of one-carbon metabolism have shown to be very flexible (Brosnan et al., 2004; Holm et al., 2007; Niculescu and Zeisel, 2002; Ueland et al., 2005), making it difficult to explain the intra-individual variation.

4.4 RISK ASSESSMENT

The distributions of arsenic in drinking water in the different countries are shown in **Fig. 9**. In the Bangladeshi population over 40% of the individuals drank water exceeding Bangladesh's drinking water standard of 50 µg/L (BWSPP, 2007) and more than 50% drank water exceeding WHO's and European Union's guideline value of 10 µg/L (European-Commission, 1998; WHO, 2003). In Hungary over 50% of the individuals consumed water containing arsenic above the European Union's standard

value, however, only two individuals consumed water containing more than 50 µg/L. In Romania and Slovakia approximately 7% and 4% consumed water exceeding European Union's standard, respectively. We conclude that mitigation activities are particularly needed in Bangladesh, but also in Europe, especially in Hungary.

As previously mentioned, inorganic arsenic is classified by IARC in Group 1, meaning that there is sufficient evidence for carcinogenicity in humans. The most recent risk assessment performed by NRC estimated the lifetime risks for bladder and lung cancer for US populations exposed to 10 µg/L in drinking water are 12 and 18 per 10,000 individuals, respectively, for females and 23 and 14 per 10,000 individuals, respectively, for males (NRC, 2001). This risk assessment is based on a linear extrapolation of cancer risks reported from studies with higher concentrations. As arsenic has been shown to have limited ability to induce point mutations some concerns have been expressed about the validity of such an extrapolation. On the other hand, the mode of action is not yet fully understood and there is increasing evidence of effects in the low dose range with epigenetic effects (Reichard et al., 2007; Singh and DuMond, 2007) as well as endocrine disruption (Benbrahim-Tallaa et al., 2005; Benbrahim-Tallaa et al., 2007; Davey et al., 2007). Furthermore, results from the case-control study in Hungary, Romania and Slovakia suggest an association between long-term arsenic exposure at low concentrations and basal cell carcinoma, bladder cancer and kidney cancer (Fletcher et al., 2006).

Other uncertainties in NRC's risk assessment could be addressed. The studies that the risk assessment was based on had limited information about individual exposure and there were also concerns about the health and nutritional status of the exposed populations. The present study clearly shows that arsenic in drinking water is not an optimal exposure marker, however, as discussed previously it is the only available marker for long term exposure. Furthermore, this study shows that the contribution of arsenic from food is significant, especially in the low dose range. These uncertainties in the exposure assessment could lead to an underestimation of the risk if the food contains inorganic arsenic and should be taken into consideration in future risk assessments.

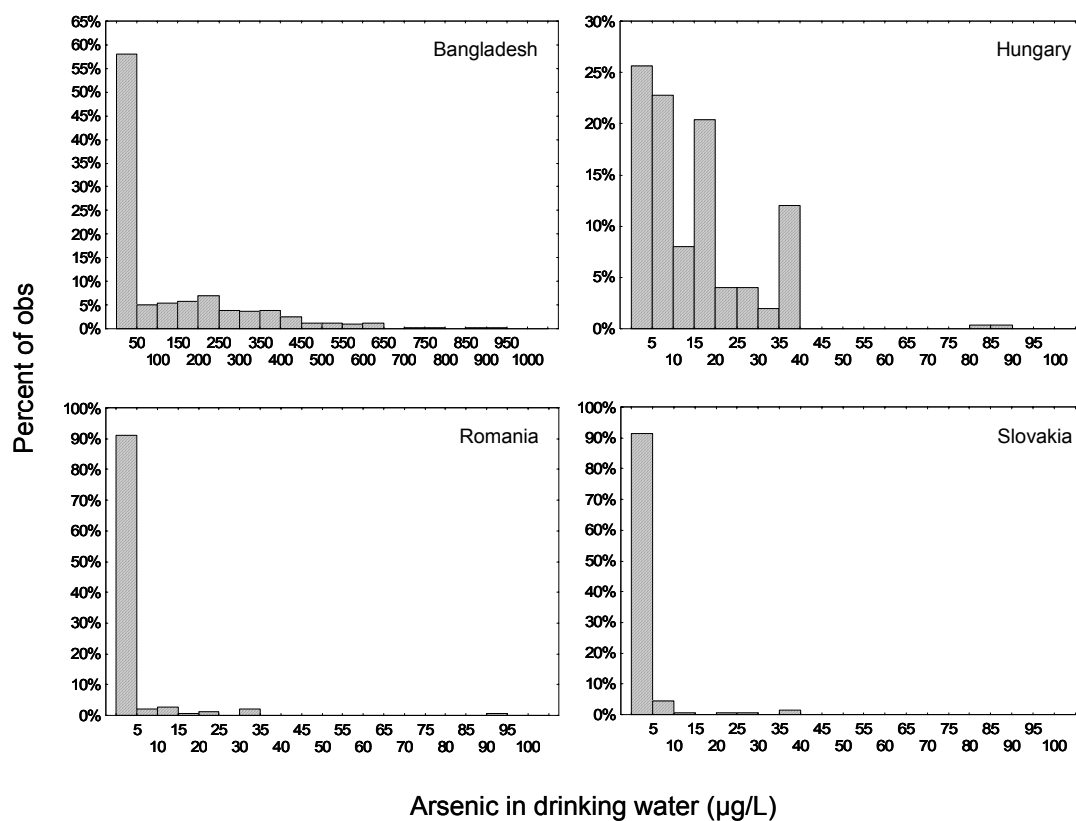


Figure 9. Distribution of arsenic concentrations in drinking water in Bangladesh (**Paper IV**), Hungary, Romania and Slovakia (**Paper II**). Two individuals from Bangladesh with 1015 µg/L and 3644 µg/L, respectively, in their drinking water were excluded.

Furthermore, as previously discussed, the methylation efficiency of arsenic has been associated with the risk of several arsenic induced toxic effects and should therefore also be taken into consideration in the risk assessment. Evaluation of factors influencing arsenic metabolism could lead to identification of susceptible subgroups. The present study shows that men had lower methylation efficiency than women and could thereby indicate that they have an increased risk compared to women, especially in certain age groups. In fact, men have been shown to be more affected by arsenic related skin effects than women (Rahman et al., 2006a). SNPs in genes coding for enzymes involved in arsenic metabolism could probably also lead to an inter-individual variation in susceptibility. As a lot of new knowledge has been added since 2001 when NRC's risk assessment was performed maybe it is time for a re-evaluation of the drinking water guidelines for arsenic.

5 CONCLUSIONS

The study found that most of the individuals in the Matlab area, Bangladesh and a large part of the individuals in Békés, Csongrad and Jasz-Nagykun-Szolnok counties in Hungary drank water containing inorganic arsenic concentrations above the drinking water standard of 10 µg/L set by WHO. In addition, there was a significant contribution of arsenic from food in both study areas.

Large inter-individual variations in the distribution of inorganic arsenic and its metabolites in urine were found within the two different populations. In Bangladesh, where the exposure was higher than in Europe, exposure level of arsenic was found to be the strongest influencing factor on arsenic metabolism. In Europe, polymorphisms in the AS3MT and MTHFR genes were found to strongly influence arsenic metabolism. Additional determinants of arsenic metabolism included gender, age and smoking. Nutritional status, however, had a small impact on arsenic methylation. The determinants studied explained approximately 20% of the inter-individual variation and evidently several other factors have a large impact on the metabolism of inorganic arsenic.

HPLC-HG-AFS was found to be a good alternative to HPLC-HG-ICPMS for speciation analysis of arsenic in urine in highly exposed areas where the requirements of low detection limits are not needed.

6 FUTURE RESEARCH

The most important task in relation to arsenic exposure is to find strategies to stop the exposure. Unfortunately, this is likely to take time and population groups may still be exposed during a transitional period. In the interim it is of great importance to identify susceptible subgroups in order to decrease the negative consequences of arsenic exposure. The present work has contributed to an increased understanding of how various factors influence arsenic metabolism, but there are of course many areas that need further research. For example (not in order of priority):

- Further research on identifying additional enzymes involved in the metabolism of arsenic.
- Large scale studies that continue to study the influence of polymorphisms on arsenic metabolism in several different populations.
- Studies on the influence of sex hormones on arsenic metabolism are needed to confirm the indications seen in the present study.
- Evaluation of the impact of the factors seen to influence the metabolism of arsenic on adverse health outcomes in order to identify susceptible subgroups.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Arsenik i dricksvatten förekommer i vissa delar av världen och kommer från naturliga geologiska källor. När brunnar borrar för vattenförsörjning lakas arseniken ut i grundvattnet. Miljontals människor runtom i världen exponeras för höga halter av arsenik via dricksvatten. Arsenik är cancerframkallande och kan efter många års exponering ge tumörer i hud, lunga, urinblåsa och njure. Arsenik har även visats kunna bidra till uppkomst av diabetes, hjärtkärlsjukdomar och effekter på lungor, lever och nervsystem. De tidigaste hälsoeffekterna av arsenik visar sig på huden i form av pigmenteringsförändringar och förtjockning av huden främst på handflator och fotsulor. Arsenik omvandlas i kroppen och utsöndras sedan till största del via urin. Urin innehåller huvudsakligen två olika omvandlingsprodukter, dels monometylerad arsenik (MA) som produceras i första steget och dels dimetylerad arsenik (DMA) som är slutprodukten. Det finns stora skillnader i hur effektivt arseniken omvandlas i kroppen, både mellan olika populationer och mellan individer. Studier har visat att personer i norra Argentina och Chile är väldigt effektiva att omvandla arseniken medan personer i Taiwan inte är det. Denna markanta skillnad i omvandlingskapacitet mellan populationer och individer kan bero på många faktorer som till exempel; ålder, kön, exponeringsnivåer, nutrition och genetiska faktorer. Flera forskningsrapporter har indikerat att en hög andel MA i urin är förknippat med en ökad risk för hälsoeffekter orsakade av arsenik.

Detta projekt syftar därför till att klargöra vilka faktorer som påverkar omvandlingskapaciteten av arsenik hos människa. Vi har mätt halterna av arsenik i dricksvatten och dess omvandlingsprodukter i urin i två olika populationer, en från Centraleuropa (Ungern, Rumänien och Slovakien) och en från Bangladesh.

Resultaten i denna avhandling visar att de flesta människorna i Matlab området i Bangladesh och en stor del av människorna i vissa delar av Ungern var exponerade för höga halter arsenik via dricksvatten. Vi kunde även visa att födan var en bidragande exponeringskälla för arsenik. Denna studie visar att det finns en markant skillnad i omvandlingskapaciteten mellan individer i både Europa och Bangladesh. I Bangladesh där exponeringen var högre än i Europa påverkades omvandlingskapaciteten mest av exponeringsnivån av arsenik. I Europa var det olika genetiska faktorer som hade störst inverkan. Övriga faktorer som påverkade omvandlingskapaciteten av arsenik var kön,

ålder och rökning. De faktorer som studerats i denna avhandling förklarade ungefär 20 % av variationen mellan individerna vilket tydligt visar att det är betydligt fler faktorer som påverkar omvandlingskapaciteten av arsenik.

Den viktigaste uppgiften i framtiden är självklart att hitta strategier för att få exponeringen av arsenik att upphöra. Tyvärr kommer detta att ta tid och det är därför viktigt att försöka identifiera känsliga grupper i befolkningen för att minska de negativa konsekvenserna av arsenikexponering. Denna avhandling har bidragit till en ökad insikt för hur olika faktorer påverkar omvandlingen av arsenik i kroppen, men det finns självklart fortfarande många frågor som behöver besvaras och det finns ett stort behov av framtida forskning inom detta område.

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