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DEVELOPMENTAL IMMUNOTOXICITY OF EARLY-LIFE ARSENIC EXPOSURE

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Developmental immunotoxicity of early-life arsenic exposure

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Arsenic exposure, mainly via drinking water and certain food, is a major public health concern because of its association with various types of cancers and other non-cancer adverse health effects. Worldwide, more than one hundred million people are exposed to elevated levels of arsenic on a regular basis. Bangladesh is one of the most severely affected countries, due to the natural occurring arsenic contamination in ground water that is used as drinking water. Although numerous studies have focused on investigating arsenic carcinogenicity, growing evidence indicates that arsenic may also affect the immune system. However, the specific effects of arsenic on immune function, particularly on the developing immune system in humans, are poorly understood.

The overall aim of the present thesis was to elucidate potential effects of arsenic exposure on the developing immune system, including underlying mechanisms and critical windows of exposure. The four papers included in the thesis were nested into a randomized, community-based food and micronutrient supplementation trial, the Maternal and Infant Nutrition Interventions, Matlab ('MINIMat') trial, conducted in Matlab, a rural area of Bangladesh. Mother-child pairs were followed from early gestation to 9 years postpartum. More than 95% of the population in the study area use groundwater, retrieved from hand pumped tube-wells, as their main drinking water. Screening of all functioning tube-wells showed a wide range of arsenic contamination; about 70% exceeded the WHO guideline value of 10 µg/L, and 50% exceeded the national standard of 50 µg/L in drinking water.

Arsenic exposure in the present studies was assessed based on the concentrations of the sum of inorganic arsenic and its methylated metabolites (monomethylarsonic acid and dimethylarsinic acid) in maternal urine during pregnancy and child urine at 4.5 and 9 years of age (Urinary arsenic: U-As), using high-performance liquid chromatography online with hydride generation and inductively coupled plasma mass spectrometry (HPLC-HG-ICPMS). In a sub-sample, we measured arsenic concentrations in maternal blood, placenta, and cord blood using ICPMS after acid digestion. Immune and inflammatory markers were measured in placenta (number of T cells, 8-oxoguanine (8-oxoG), cytokines, and leptin) and cord blood (cytokines, signal joint T cell receptor excision circles (sjTRECs), 8-hydroxy-2'-deoxyguanosine (8-OHdG), oxidative-stress defense and apoptosis related genes). Cell-mediated immunity was measured by delayed type hypersensitivity in response to purified protein derivative (PPD) injection and plasma cytokines were measured in children at 4.5 years of age. Plasma total IgG and measles-mumps-rubella vaccination-specific IgG concentrations were measured in children at 9 years of age.

The median U-As concentration was 77 µg/L (range 2-2,064 µg/L) during early pregnancy, 57 µg/L (range 12-1,228 µg/L) in children at 4.5 years, and 53 µg/L (range 9-1,268 µg/L) at 9 years of age. The concentrations at different time points were significantly correlated, indicating somewhat reduced exposure over time. Arsenic exposure in early pregnancy was associated with reduced number of T cells in the placenta. Maternal arsenic exposure was

also associated with decreased thymic function (number of naïve T cells) in newborns (measured by sjTREC_s in umbilical cord blood lymphocytes), particularly in response to maternal infections. Maternal U-As was positively associated with oxidative stress markers (8-oxoG in placenta and 8-OHdG in cord blood). Oxidative stress responsive genes were down-regulated and apoptosis related genes were up-regulated in cord blood in relation to elevated arsenic concentrations. Placental inflammatory cytokines (IL-1 β , TNF- α , and IFN- γ) and leptin increased with increasing maternal arsenic exposure. Cord blood cytokines (IL-1 β , IL-8, IFN γ , and TNF α) showed a U-shaped association in relation to maternal arsenic exposure. Thus, prenatal arsenic exposure seems to impair vital immune responses in newborns.

In children at 4.5 years of age, arsenic exposure was related to a weak response to the injected purified protein derivative. Particularly, the association was stronger in children with recent infections, indicating that arsenic impaired T cell-associated immune response to infections. In addition, the children's arsenic exposure was associated with reduced plasma concentrations of Th1 cytokines (IL-2 and TNF- α) without alteration of the Th2 cytokines. In children at 9 years of age, prenatal arsenic exposure was positively associated with plasma total IgG, and the association was apparent mainly in boys and in malnourished children. Furthermore, we also found preliminary evidence of decreasing mumps-specific vaccination response with increasing arsenic exposure during childhood.

In summary, elevated arsenic exposure appeared to adversely affect the developing immune system, including both T cell and B cell-associated immune functions. Maternal arsenic exposure seemed to reduce T cells in placenta and cord blood and increase nonspecific antibody production by B cells in children at 9 years of age. Early childhood arsenic exposure appeared to reduce cell-mediated immune function and vaccination response. These effects may have consequences for susceptibility to various immune-related diseases later in life, and thus these findings are of high public health relevance since the main source of arsenic exposure is via drinking water and food. Therefore, more effective mitigation strategies for arsenic exposure are needed.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-IV:

- I. **Ahmed S**, Mahabbat-e Khoda S, Rekha RS, Gardner RM, Ameer SS, Moore S, Ekström EC, Vahter M, Raqib R (2011). Arsenic-associated oxidative stress, inflammation, and immune disruption in human placenta and cord blood. *Environ Health Perspect* 119(2), 258-64.
- II. **Ahmed S**, Ahsan KB, Kippler M, Mily A, Wagatsuma Y, Hoque AM, Ngom PT, El Arifeen S, Raqib R, Vahter M (2012). *In utero* arsenic exposure is associated with impaired thymic function in newborns possibly via oxidative stress and apoptosis. *Toxicol Sci* 129(2), 305-14.
- III. **Ahmed S**, Moore SE, Kippler M, Gardner R, Hawlader MD, Wagatsuma Y, Raqib R, Vahter M (2014). Arsenic exposure and cell-mediated immunity in pre-school children in rural Bangladesh. *Toxicol Sci* 141(1), 166-75.
- IV. **Ahmed S**, Ahsan KB, Kippler M, Lu Y, El Arifeen S, Wagatsuma Y, Vahter M, Raqib R (2014). Plasma IgG and vaccination response in arsenic-exposed children in rural Bangladesh. *Manuscript*.

LIST OF RELATED SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

- I. Raqib R, **Ahmed S**, Sultana R, Wagatsuma Y, Mondal D, Hoque AM, Nermell B, Yunus M, Roy S, Persson LA, Arifeen SE, Moore S, Vahter M (2009). Effects of *in utero* arsenic exposure on child immunity and morbidity in rural Bangladesh. *Toxicol Lett* 185(3), 197-202.
- II. **Ahmed S**, Rekha RS, Ahsan KB, Doi M, Grander M, Roy AK, Ekstrom EC, Wagatsuma Y, Vahter M, Raqib R (2013). Arsenic exposure affects plasma insulin-like growth factor 1 (IGF-1) in children in rural Bangladesh. *PLoS One* 8(11), e81530.
- III. Broberg K, **Ahmed S**, Engstrom K, Hossain MB, Jurkovic Mlakar S, Bottai M, Grander M, Raqib R, Vahter M (2014). Arsenic exposure in early pregnancy alters genome-wide DNA methylation in cord blood, particularly in boys. *J Dev Orig Health Dis* 5(4), 288-98.

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LIST OF ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
8-oxoG	8-oxoguanine
µg/L	Micrograms per liter
Amu	Atomic mass unit
As(III)	Arsenite
As(V)	Arsenate
BMI	Body mass index
CBMC	Cord blood mononuclear cells
CD	Clusters of differentiation
CHRW	Community health research worker
CV	Coefficient of variation
DIT	Developmental immunotoxicity
DMA(III)	Dimethylarsinous acid
DMA(V)	Dimethylarsinic acid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FcγRI	Fc-gamma receptor 1
GPx	Glutathione peroxidase
GW	Gestational week
HDSS	Health and demographic surveillance system
HG	Hydride generation
HPLC	High-performance liquid chromatography
iAs	Inorganic arsenic
IARC	International Agency for Research on Cancer
Icddr, b	International Centre for Diarrheal Disease Research, Bangladesh
ICPMS	Inductively coupled plasma mass spectrometry
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin

MHC	Major histocompatibility complex
MINIMat	Maternal and infant nutrition interventions in Matlab
MMA(III)	Monomethylarsonous acid
MMA(V)	Monomethylarsonic acid
MMR	Measles, mumps, and rubella
MPO	Myeloperoxidase
NK cells	Natural killer cells
NOS	Nitric oxide synthase
nTreg	Natural regulatory T cells
PBMC	Peripheral blood mononuclear cells
PPD	Purified protein derivative
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SAM	S-adenosylmethionine
SjTREC	Signal joint T cell receptor excision circle
SES	Socioeconomic status
SG	Specific gravity
SOD	Superoxide dismutase
TCR	T cell receptor
Th1	T helper type 1
Th2	T helper type 2
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor alpha
tIgG	Total plasma IgG
U-As	Urinary arsenic, sum of iAs, MMA and DMA
UNICEF	United Nations Children's Fund
WHO	World Health Organization

1 INTRODUCTION

The present thesis focuses on immune effects of arsenic exposure, particularly exposure early in life. The immune system is an integrated complex network of organs, tissues, cells, and cell products which protects the body from pathogens and tumor cells. Since the immune system develops through a series of pre- and postnatal events, the developing immune system is more sensitive to toxic insults than the adult immune system (Dietert 2008). There is increasing evidence that early life exposures to environmental pollutants may cause chronic diseases later in life (Godfrey and Barker 2000; Langley-Evans 2006). Arsenic is a potent human toxicant and carcinogen, but knowledge is limited about its effects on the developing immune system. Inorganic arsenic and its methylated metabolites can easily cross the placenta (Concha et al. 1998). Several epidemiological studies have indicated an increased risk of infectious diseases in children in relation to maternal arsenic exposure during pregnancy (Raqib et al. 2009; Vahter 2009; Rahman et al. 2011; Farzan et al. 2013; Farzan et al. 2013). Arsenic exposure early in life may affect the immune system and contribute to later life effects (Smith et al. 2006; Vahter 2009). Thus, it is important to elucidate potential effects of arsenic exposure on the developing immune system in humans.

1.1 IMMUNE RESPONSE

The immune response is a complex array of defense mechanisms which involve protecting a host from pathogenic infection and malignant cells (Abbas et al. 2012). The central role of the immune system is to distinguish between the host and foreign invaders and to control and clear those foreign invaders. The immune system has two separate responses, the innate and the adaptive immune responses, which function synergistically to fight against infection. The first line of defense is the innate immune response, which is activated within few hours of infection in an attempt to inhibit and control the invading pathogen. It either eliminates the pathogen or halts it until the adaptive immune response has been activated. The innate immune response includes epithelial barriers and antimicrobial peptides present in the epithelia, complement proteins, and immune cells such as macrophages, neutrophils, natural killer cells, dendritic cells, mast cells, eosinophils, and basophils. The phagocytic cells migrate to the site of infection and destroy the pathogens by engulfing them. Cytokines and chemokines are released from these immune cells and facilitate cell-to-cell communication by functioning as chemical messengers. The second line of immune defense is the adaptive immune response, which mainly consists of T lymphocytes and B lymphocytes, and their products, such as antibodies. Antigen presenting cells, particularly macrophages and dendritic cells, process pathogens and present antigen (molecule or molecular fragment that can provoke a targeted antibody response) using major histocompatibility complex II (MHCII) molecules to CD4+ T lymphocytes which then become activated upon of the MHC-antigen complex by the T cell receptor. Depending on the cytokine environment, the activated T cells either migrate to the infection site to initiate cell-mediated immunity or migrate to the lymphoid organs to activate B lymphocytes. Activated mature B cells form plasma cells that

are accountable for the production of antigen-specific antibodies. B cells and T cells are also converted to memory cells to establish immune memory for a particular antigen, leading to a boosted and more rapid response at subsequent encounters with the same pathogen. This feature of adaptive immunity is the basis of vaccination.

1.1.1 The lymphoid organs

The lymphoid organs are sites where lymphocytes are produced, matured, and are presented with antigen by innate immune cells. They are divided into primary and secondary lymphoid organs. Bone marrow and thymus are the primary lymphoid organs, where differentiation, proliferation, and maturation of stem cells take place to become immune competent lymphocytes. During fetal life, the primary immune organs are initially the yolk sac, then the spleen and fetal liver, and finally the bone marrow and thymus (Nairn and Helbert 2002). The secondary lymphoid organs are spleen, lymph nodes, tonsils, appendix, peyer’s patches, and various lymphoid tissues, including bronchus-associated lymphoid tissue and gut-associated lymphoid tissue (Cyster 2003). These are sites, where lymphocytes are lodged, are presented with antigen via their specific antigen receptors, and expanded clonally.

1.1.1.1 The bone marrow

The mammalian bone marrow is the site of hematopoiesis and maturation of B cells. Innate and adaptive immune cells are generated from the hematopoietic stem cells in the bone marrow (Shaikh and Bhartiya 2012). Common myeloid and lymphoid progenitors originating from the hematopoietic stem cells mature into different cell types (**Figure 1**) by the influence of growth factors and cytokines (Shaikh and Bhartiya 2012). The precursor of T cells also originates from the bone marrow, but the final development is completed in the thymus (Yang et al. 2010).

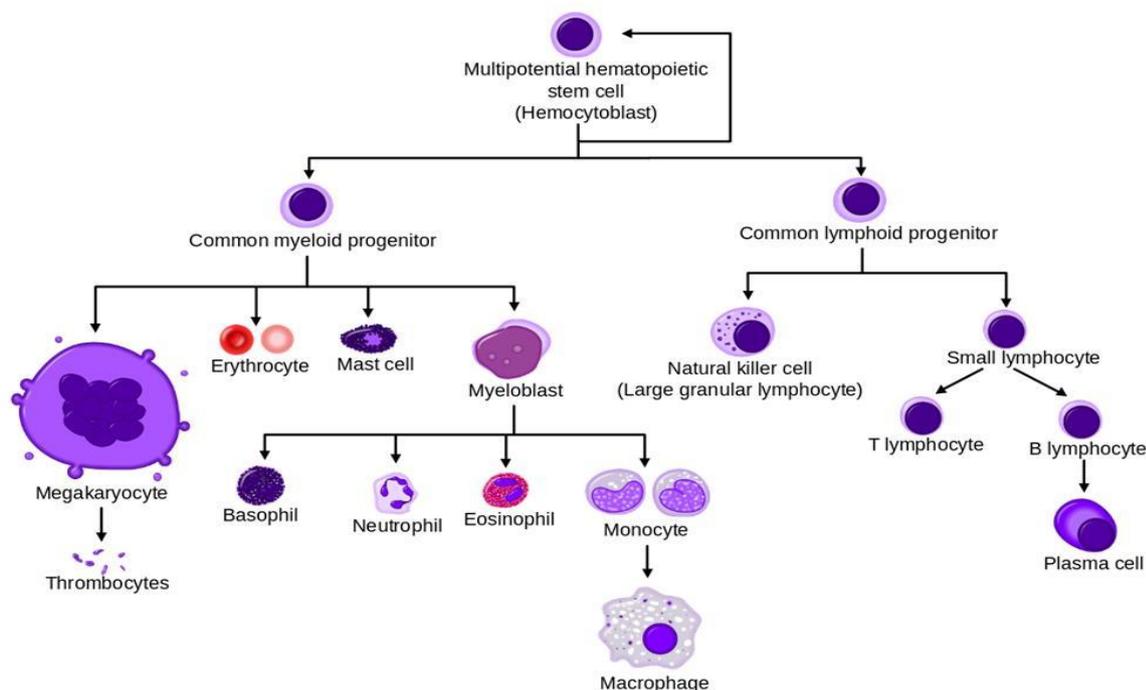


Figure 1: Hematopoiesis in bone marrow [adapted from (Shaikh and Bhartiya 2012)].

1.1.1.2 The thymus

The thymus is the site of T cell development, maturation, and selection. It is a bi-lobed organ, located in the anterior mediastinum of the thoracic cavity. Each lobe of the thymus has two compartments; outer cortex and inner medulla. The outer cortex is densely packed with immature T cells (thymocytes) while the inner medulla is sparsely populated with mature T cells (Nishino et al. 2006). Hematopoietic lymphoid progenitor cells enter the thymus to become selected as functional T cells. In the thymic cortex, progenitor cells mature to CD4+ CD8+ double positive cells, and then T cell receptor gene rearrangements (TCR) occur so that the mature T cells can collectively recognize a wide variety of antigens. Thymocytes those are unable to make appropriate gene rearrangements undergo apoptosis or programmed cell death. Thymocytes with an appropriately expressed TCR undergo positive and negative selection in thymic cortex and medulla, respectively. In the positive selection, TCRs on the developing thymocytes interact with thymic epithelial cells in the cortex via MHC molecules, and those with no affinity are destroyed. In the negative selection, thymocytes come in contact with the thymic dendritic cells in the medulla, where upon those showing higher affinity to the thymic dendritic cells are eliminated through apoptosis (to avoid autoimmunity). This multi-step process ensures strict selection during which only 1-3% of the thymocytes become functional T cells, which are thereafter exported from the thymus as CD4+ or CD8+ T cells (Taub and Longo 2005). The thymus continues to grow until puberty and then it begins to atrophy and the organ is primarily replaced with fatty tissues, a process known as thymic involution. As the immune system ages, fewer naïve T cells are produced, which can lead to consequently impaired cell-mediated immune function. This is probably due to thymic involution (Taub and Longo 2005).

1.1.2 Cell-mediated immune response

Cell-mediated immunity provides defense against intracellular microbes and transformed cells (Peter J. Delves 2011; Abbas et al. 2012). When T cells encounter antigens by antigen-presenting cells through MHC I or MHCII molecules, they become activated. Activated CD4+ cells secrete different kinds of cytokines, depending on the type of antigen, and activate phagocytes that kill microbes which have been internalized into intracellular vesicles. The activated CD8+ cytotoxic T cells kill host cells that are harboring microbes or presenting tumor antigens.

1.1.3 Cytokines

Cytokines are soluble proteins, produced by immune cells in small quantities in response to external stimuli that bind to high-affinity receptors on their target cells. Cytokines can function in an autocrine (same cell), paracrine (adjacent cells), or endocrine (enter the circulation) fashion. When antigen presenting cells present their antigen to naïve CD4+ T cells by MHC class II molecules, CD4+ cells are converted into either T helper type 1 (Th1) or T helper type 2 (Th2) cells, depending on the immune response and the cytokine environment. The Th1 cells predominantly secrete IL-2, TNF- α , and IFN- γ , and are associated with cell-mediated immunity. IL-2 stimulates the proliferation of T cells, whereas

TNF- α and IFN- γ stimulate macrophages to kill the intracellular pathogens (Berger 2000). The Th2 cells mainly produce IL-4, IL-5, IL-10, and IL-13, which stimulate B cells to produce antigen-specific antibodies.

1.1.4 Humoral immune response

Humoral immune response is mediated by antibodies that neutralize and eliminate extracellular microbes and their toxins. The naïve B cells are activated by antigens through T cell-dependent or T cell-independent pathways and become effector cells (plasma cells) that actively secrete antigen-specific antibodies. B cells also internalize the antigens and present them to the antigen-specific T cells, which stimulate more naïve B cells to convert into plasma cells (Abbas et al. 2012). T cells can only recognize protein antigens whereas B cell produced antibodies can recognize different protein, carbohydrate and lipid antigens.

1.2 DEVELOPMENTAL IMMUNOTOXICITY (DIT)

The development of the immune system occurs mainly prenatally with important maturation steps occurring after birth. In early pregnancy, the maternal immune system shifts towards Th2 cytokine response in order to protect the fetus from being recognized as foreign (Calleja-Agius and Brincat 2008). As a result, the Th1-dependent cytokine production is suppressed. Immaturity of dendritic cells and macrophages probably due to the action of placental growth factor or 17- β estradiol, increased production of certain Th2 cytokines, and down regulation of IFN- γ are the hallmarks of normal pregnancy state (Dietert and Zelikoff 2008). Regulatory T cells (Zenclussen et al. 2007) and NK cells (Saito et al. 2007) help to maintain a local environment where inappropriate allogeneic antigen stimulation is minimized. Thus, the child is born with Th2 predominant immune responses. After birth, the immune system requires further maturation to develop adequate Th1 cytokine responses which are required for an effective host resistance against infections (Dietert and Zelikoff 2008). The maturation of this Th1 cytokine response in children is achieved by further development of dendritic cells, macrophages, and other immune cells. These series of pre- and postnatal events seem to be more sensitive to toxic insult than the adult immune system (Dietert 2009). Developmental immunotoxicity (DIT) is defined as toxicant-induced unbalanced immune maturation during the pre- and postnatal period, which may be manifested in a state of immunosuppression, nonspecific immunostimulation, hypersensitivity, or autoimmunity. DIT is a novel research area which is gaining increasing recognition as a significant factor for a variety of childhood and later life diseases. For example, maternal smoking and intake of alcohol during pregnancy have been shown to cause immune dysfunction (Dietert and Zelikoff 2008).

1.2.1 Developing immune system is uniquely sensitive to toxicants

During the pre- and postnatal period, development of the immune system progresses through a well-defined series of cellular and organ events. In humans, the time from conception through two years of age is considered the most vulnerable period to toxicants (Dietert and Zelikoff 2008). The processes most susceptible to DIT are seeding of the thymus with lymphoid progenitor cells, positive and negative selection of thymocytes, and maturation of

dendritic cells (Dietert and Piepenbrink 2006). A developmental vulnerability may be revealed as a qualitative difference; toxicants may only affect the developing immune system whereas the adult immune system remains unaffected (**Figure 2A**). Toxic effects of a xenobiotic may not be identified if immune function is only evaluated in adults. The vulnerability may also be manifested as a quantitative difference, where a lower dose is required to affect the developing immune system as compared to the adult immune system (**Figure 2B**). Finally, the developmental vulnerability may be revealed as a temporal difference, where the effect is more persistent in younger individuals than in adults (**Figure 2C**) (Luebke et al. 2006; Burns-Naas et al. 2008). Multiple studies examining a range of xenobiotics have indicated that the developing immune system is hypersusceptible to toxicants when compared with that of the adults (Dietert et al. 2000; Dietert and Piepenbrink 2006; Luebke et al. 2006; Dietert 2008). For example, polychlorinated biphenyl (PCB)-related dioxin exposure in rodents, there seems to be an approximately 100-fold difference in dose sensitivity in developing immune system than that of adult (Luebke et al. 2006). Studies have shown that both prenatal and childhood exposure to PCB is associated with reduced antibody response to childhood vaccination and an increased risk of infections (Weisglas-Kuperus et al. 2000; Heilmann et al. 2006).

Early life exposure to several heavy metals has been associated with developmental immunotoxicity (Dietert and Zelikoff 2008). Maternal mercury exposure during pregnancy decreased cord blood naïve T cells and reduced T cell proliferation in response to mitogen (Belles-Isles et al. 2002). Children's blood lead levels were found to be associated with decreased CD4+ T cells and increased plasma concentration of IgE, which indicated imbalanced Th1 and Th2 cytokine responses (Sun et al. 2003; Li et al. 2005).

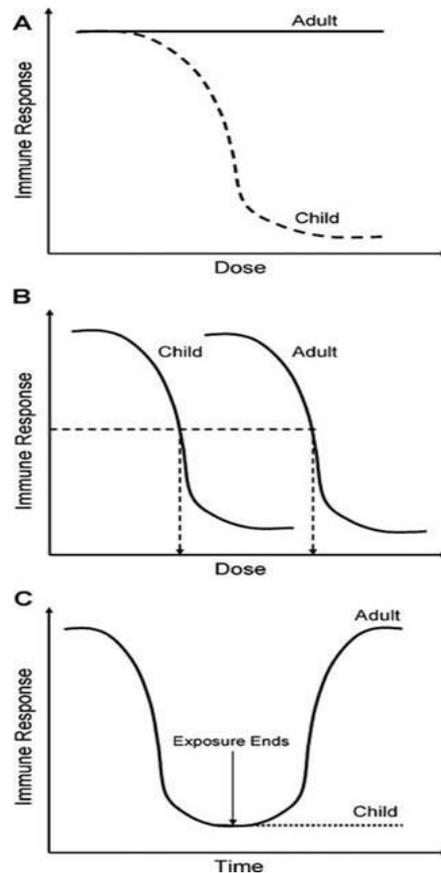


Figure 2: Susceptibility to toxicants in children compared with adults [adapted from (Burns-Naas et al. 2008)].

1.3 ARSENIC

Arsenic is an element with atomic number 33 and a relative atomic mass, 74.92 amu. It is often referred to as a metalloid because of its chemical and physical properties, which are intermediate between metals and non-metals. Arsenic is found in numerous minerals, usually in combination with sulfur and other metals. In reducing and oxygenated conditions, arsenite (AsIII), and arsenate (AsV), are the main oxidation states, respectively (WHO 2001; IARC 2004). Compounds of arsenic are divided into three major groups.

- inorganic arsenic compounds (arsenic trioxide, sodium arsenite, arsenic trichloride, arsenic acid, and arsenic pentoxide),

- organic arsenic compounds (arsanilic acid, methylarsonic acid, dimethylarsinic acid, arsenobetaine and arsenosugars), and

- arsine gas.

Arsenite and arsenate are the most common inorganic forms in water, while arsenobetaine, arsenocholine, and arsenosugars are the important organic forms in certain foods, particularly in sea foods (IARC 2004).

1.3.1 Use of arsenic

Current and historical uses of arsenic and arsenic compounds are in wood preservatives, agricultural chemicals, pharmaceuticals, and applications in the mining, semiconductor, and glass-making industries. Organic arsenicals (e.g. roxarsone, arsanilic acid, and its derivatives) are used to increase the rate of weight gain for poultry and swine (Silbergeld and Nachman 2008). In the manufacture of alloys, metallic arsenic is used, mostly with lead (e.g. in lead acid batteries) and copper. Because of the light-emitting and electromagnetic properties of gallium arsenide, it is used in high-speed semiconductor devices (IARC 2006). In computer chips and fiber optics, arsine gas is used as a doping agent (IARC 2012).

1.3.2 Environmental arsenic

Arsenic is ubiquitous, and it is the 20th most common element in the earth's crust in relation to other elements. In nature, arsenic is mostly present as a mineral arsenopyrite. Arsenic is released from the earth's crust to the environment as a result of volcanic and industrial activities. Mining, smelting, and the burning of fossil fuels are the main anthropogenic sources of arsenic contamination of water, soil, and air. The ambient environment has also been contaminated by the historical use of arsenic containing pesticides (WHO 2001). Arsenic is mainly transported in the environment via water from both natural and anthropogenic sources. In some regions of the world, groundwater (used for drinking water) is naturally contaminated with arsenic due to arsenic rich geological formations. These areas include Bangladesh, China, Taiwan, West Bengal (India), and some part of Argentina, Chile, Mexico, Vietnam, Australia, and the USA. In unaffected areas, the levels of arsenic are only a few micrograms per liter of ground water, whereas, in affected areas, the levels may range from tens to thousands of micrograms per liter (IARC 2012).

1.3.3 Human exposure

Ingestion of arsenic contaminated water is the primary route of inorganic arsenic exposure for the general population. More than one hundred million individuals world-wide are at risk of elevated arsenic exposure, mainly via drinking water. The situation is particularly problematic in Bangladesh and West Bengal, where a substantial fraction of many millions of hand-pumped tube-wells yield drinking water with arsenic concentrations above 10 µg/L (Kinniburgh and Smedley 2001), the drinking water guideline value of the World Health Organization (WHO 2004). Millions of people in the USA, China, South America and Europe are also currently exposed to arsenic concentrations above 10 µg/L in drinking water (IARC 2004; Mukherjee et al. 2006; Vahter 2009).

Food, such as rice, is an increasingly important source of exposure to inorganic arsenic, particularly in areas where arsenic-rich groundwater is used for irrigation (Zhu et al. 2008; Adomako et al. 2009; Rahman and Hasegawa 2011). People may also be exposed to arsenic via seafood, which mainly contains organic forms of arsenic, such as arsenobetaine, arsenosugars, and arsenocholine. These forms are considered to be less toxic than inorganic

arsenic. Finally, people may be exposed via inhalation of arsenic-containing particulates in areas with industrial emissions (Wang et al. 2009).

1.3.4 Arsenic exposure in Bangladesh

Historically, the surface water used for drinking water in Bangladesh was contaminated with microorganisms, which caused a significant burden of morbidity and mortality due to gastrointestinal diseases, particularly among infants and children. By installation of millions of tube-wells, the country has nearly achieved the target of providing pathogen-free water. The installation of tube-wells began during the 1970s, and it was conducted by the Bangladesh Department of Public Health Engineering with the help of United Nations Children's Fund (UNICEF) along with other aid agencies (Smith et al. 2000). The presence of arsenic in tube-well water, at concentrations above the local drinking water standard (≥ 50 $\mu\text{g/L}$), was initially reported in the early 1990s (Nickson et al. 1998). Presently, in rural areas of Bangladesh, 97% of the population relies on tube-well water as their main drinking water source (Flanagan et al. 2012). According to survey data, it is estimated that about 35-77 million people are chronically exposed to high levels of arsenic (>10 $\mu\text{g/L}$, WHO drinking water guideline value) in their drinking water (Winston et al. 2013). As groundwater is used during the dry season for irrigation of the fields, people are additionally exposed to inorganic arsenic via food, such as rice, which is the staple food in Bangladesh.

1.3.5 Absorption and metabolism of arsenic

Approximately 80-90% of ingested inorganic arsenic is absorbed in the gastrointestinal tract both in humans and animals (Vahter and Norin 1980; Marafante et al. 1987). Absorbed arsenate is first reduced in the blood (Vahter and Envall 1983), and then methylated, mainly in the liver. In humans, inorganic arsenic is metabolized through the conversion of AsV to AsIII, followed by methylation to monomethylated and dimethylated arsenicals (MMA and DMA, respectively) (Vahter 2009; Watanabe and Hirano 2013). The one carbon metabolism component S-adenosylmethionine (SAM) acts as the main methyl donor (Marafante and Vahter 1984). In presence of glutathione or other thiols, arsenic(+3)-methyltransferase (AS3MT) transfers the methyl groups from SAM to arsenic in its trivalent form (Watanabe and Hirano 2013). The main metabolites excreted in urine are DMA (60%–80% of total metabolites in urine), MMA (about 10%–20%), and some inorganic arsenic (unmethylated, 10%–30%). The sum of these metabolites is used as a measure of exposure to inorganic arsenic, and the relative proportion of each metabolite is used as a measure of methylation capacity or efficiency (Vahter 2009). Arsenic methylation was found to be affected by genetic polymorphisms in one carbon metabolism enzymes (Schlawicke Engstrom et al. 2009). MMAIII is considered the most toxic arsenical in *in vitro* and *in vivo* studies (Petrick et al. 2000; Mass et al. 2001; Vahter 2009). Individuals who excrete a higher proportion of ingested arsenic as urinary MMA, which may be directly proportional to MMAIII in tissues, is associated with an increased risk of arsenic-associated adverse health effects (Huang et al. 2008; Lindberg et al. 2008). Several studies have reported that children might have better methylation capacity than adults (Sun et al. 2007; Tseng 2009).

1.3.6 Mechanism of arsenic toxicity

Although numerous studies have examined the mechanisms of arsenic toxicity, the exact mechanisms are not yet clear. Arsenic shows its toxicity by different mechanisms depending on the dose, duration of exposure, tissue type, and metabolism. The trivalent forms of arsenic, which are highly reactive, bind preferentially to sulfhydryl (SH-) groups, resulting in inhibition of various enzymes (WHO 2001), such as DNA repair enzymes (Walter et al. 2007) and antioxidant related enzymes, e.g., glutathione peroxidase and thioredoxin reductase (Ganyc et al. 2007). Other proposed mechanisms of arsenic toxicity include immunosuppression, oxidative stress, chromosomal aberrations, micronuclei formation, modification of cellular signaling, induction of apoptosis, altered expression and DNA binding activity of transcription factors, and epigenetic modifications (NRC 2001 ; IARC 2004; Vahter 2009; Flora 2011; Dangleben et al. 2013; Farzan et al. 2013).

1.3.7 Health effects of arsenic

The International Agency for Research on Cancer (IARC) classifies arsenic as a class 1 carcinogen, as it can cause skin, lung, urinary bladder, kidney, possibly liver, and prostate cancer in humans (IARC 2012). In addition, exposure to inorganic arsenic through drinking water has been associated with increased risk of several non-cancer effects, e.g., hyperkeratosis; pigmentation changes; cardiovascular diseases including hypertension; respiratory effects; neurological, liver and kidney disorders; and diabetes mellitus (Brown and Ross 2002; IARC 2004; WHO 2004; Vahter 2009; IARC 2012; Farzan et al. 2013). The earliest effects of exposure to inorganic arsenic via drinking water include pigmentation changes in the skin and hyperkeratosis, which have been reported to appear after 5 to 10 years of exposure, and eventually cause cancer-related, as well as non-cancer, health effects (Rahman et al. 2006; Guha Mazumder and Dasgupta 2011).

Arsenic easily passes through the placenta (Concha et al. 1998). Maternal arsenic exposure during pregnancy has been related to increased risk of spontaneous abortion, stillbirth, preterm birth, and low birth weight (Rahman et al. 2007; Rahman et al. 2009; Vahter 2009; Rahman et al. 2010; Farzan et al. 2013). In addition, maternal arsenic exposure during pregnancy has also been associated with increased risk of infant mortality, child growth restriction, as well as decreased neurodevelopment of young children (Vahter 2009; Hamadani et al. 2011; Saha et al. 2012; Farzan et al. 2013).

1.3.8 Arsenic-associated immune effects

1.3.8.1 Human Studies

There is increasing evidence indicating that chronic exposure to inorganic arsenic, even at fairly low exposure levels, leads to increased risk of infectious diseases, particularly lower respiratory tract infections, diarrhea, bronchiectasis, and tuberculosis in children and adults (Smith et al. 2006; Raqib et al. 2009; Rahman et al. 2011; Smith et al. 2011; Farzan et al. 2013; Smith et al. 2013). These associations are most likely due to arsenic-related alterations

of the immune function, especially in the form of immunosuppression. A study on peripheral blood mononuclear cells from healthy subjects showed that low concentrations of arsenic (submicromolar) increased the number of natural T regulatory (nTreg) lymphocytes, whereas in apparently healthy subjects with chronic arsenic exposure, the number and function of nTreg lymphocytes appeared to decrease with increasing arsenic exposure (Hernandez-Castro et al. 2009). Approximately 10% of circulating CD4+ T cells are nTreg lymphocytes (CD25+Foxp3+ T cells) which play a significant role in maintaining immune homeostasis (Miyara and Sakaguchi 2007). In Mexican children (6-10 years of age), arsenic exposure has been associated with reduced proliferative response of peripheral blood mononuclear cells (PBMC) to mitogens, reduced percentage of CD4+ T cells, reduced CD4/CD8 ratio, and decreased IL-2 secretion without altering CD8+ proportion (Soto-Pena et al. 2006). The decreased CD4/CD8 ratio may be an early indicator of arsenic-related immunosuppression, as low CD4/CD8 is used as a surrogate marker of immunosuppression (Wikby et al. 1998). In another Mexican study, a higher rate of apoptosis in PBMC was reported in arsenic-exposed children compared to unexposed children (Rocha-Amador et al. 2011). In immune homeostasis, apoptosis is important in appropriately limiting immune responses, but abnormal immune cell apoptosis may contribute to dysregulated immune function potentially leading to immunodeficiency (Thompson 1995). Arsenic exposure may also impair the maturation, differentiation, and phagocytic function of macrophages, which have a vital role in cellular immunity, as shown in arsenic-exposed adults with skin lesions compared to unexposed individuals (Banerjee et al. 2009). Arsenic exposure may also reduce innate immune defense by reducing human β -defensin-1, an antimicrobial peptide that also acts as a tumor suppressor protein, as shown in arsenic-exposed adults in Nevada and Chile (Hegedus et al. 2008). Finally chronic arsenic exposure may alter the humoral immune response. A study conducted in Bangladesh showed significantly elevated levels of serum IgG, IgA, and IgE in arsenic-exposed adults with skin lesions, compared to unexposed adults (Islam et al. 2007).

Because inorganic arsenic and its metabolites easily pass the placenta, arsenic could potentially affect the prenatal immune maturation process. Indeed, in our previous study we have shown that maternal urinary arsenic during pregnancy is associated with reduced child thymic index, a measure of thymic volume that is functionally associated with childhood immune response, as well as reduced expression of the trophic factor interleukin-7 (IL-7), essential for thymus and T cell development, in breast milk. Maternal urinary arsenic was also inversely associated with lactoferrin, a marker of innate immunity that also functions as an antioxidant (Raqib et al. 2009).

1.3.8.2 Experimental studies

A few experimental studies have also shown adverse effects of arsenic on the immune system, in particular immunosuppression (Selgrade 2007). Mice exposed to inorganic arsenic (<100 μ g/L in drinking water) for 5-6 weeks showed down regulation of the toll-like receptor (TLR) signaling pathway and IL-1 β genes in lung tissues (Kozul et al. 2009). Studies in

rodents have shown that arsenic exposure can reduce the weight, and/or cellularity of major immunocompetent organs, including spleen and thymus (Aggarwal et al. 2008; Xia et al. 2009). *In vitro* studies have shown that arsenic exposure inhibits the formation of primary and/or secondary antibody forming cells in rodent splenocytes (Sikorski et al. 1991; Burchiel et al. 2009; Flora 2011; Nain and Smits 2012). A study in zebrafish showed that exposure to very low concentrations of arsenic (below 10 µg/L, which is considered to be safe in drinking water) resulted in a 17-fold increase in bacterial load and 50-fold increase in viral load in embryos, indicating arsenic-induced immune disruption and poorer resistance to infection (Nayak et al. 2007). In addition, inorganic arsenic significantly reduced allograft rejection in a mouse heart transplantation model compared to control, suggesting that arsenic disrupts the immune system's ability to discriminate self from non-self-antigens (Yan et al. 2009).

1.3.8.3 Conclusion

In conclusion, these data indicate that arsenic is an immunomodulator that could make the host immunocompromised. However, little is known about the effects of early-life arsenic exposure on the developing immune system in humans. Therefore, more studies should focus on the early-life arsenic exposure and evaluate the potential developmental immunotoxicity of arsenic.

2 AIMS OF THE THESIS

Despite the high prevalence of elevated arsenic exposure worldwide, most of the documented research is on arsenic carcinogenicity in humans. Few have investigated the effects of arsenic on the immune system, and even fewer on the developing immune system. Additionally, mechanisms by which arsenic may disrupt immune function are poorly understood.

The overall aim of the present thesis was to investigate potential effects of arsenic exposure on the developing immune system, including underlying mechanisms and critical windows of exposure.

Specifically, this thesis aims to elucidate:

- The effects of maternal arsenic exposure during pregnancy on immune markers in placenta and cord blood (**Paper I**).
- The effects of maternal arsenic exposure during pregnancy on thymic function in newborns (**Paper II**).
- The effects of pre- and postnatal arsenic exposure on cell-mediated immune function in children at 4.5 years of age (**Paper III**).
- The effects of pre- and postnatal arsenic exposure on humoral immune function in children at 9 years of age (**Paper IV**).

3 MATERIALS AND METHODS

This section is a summary of the materials and methods used in this thesis. For further details, the reader is referred to the individual papers (**Paper I-IV**).

3.1 STUDY AREA

The study area was Matlab (**Figure 3**), a rural sub-district, located 53 km southeast of Dhaka, the capital of Bangladesh. Matlab is situated on a flood-prone low-lying delta where the Meghna River joins to the confluent streams of two other rivers -the Brahmaputra and Ganges. The groundwater in Matlab is highly contaminated by the natural arsenic-laden soils transported by the rivers from the Himalayan Mountains in the north. More than 95% of the population uses groundwater from hand-pumped tube-wells as their drinking water (Icddr 2006). Screening of all the functioning tube-wells in Matlab for arsenic showed a wide range of concentrations (Vahter et al. 2006). About 70% of the wells exceeded 10 µg/L, the WHO guideline value for arsenic in drinking water; and 50% exceeded the 50 µg/L, the national standard for drinking water (Rahman et al. 2006). A significant proportion of inhabitants in Matlab are landless (owning no land for cultivation). The major occupations in this area are agriculture, fishing, and day labor.

In Matlab, the International Centre for Diarrheal Disease Research, Bangladesh (icddr, b) has maintained an internationally recognized and unique prospective Health and Demographic Surveillance System (HDSS) since 1966, covering 142 villages in a population of about 220,000 on 18,386 hectares of land (Icddr 2012). The area covered by HDSS is divided into two parts: the icddr,b service area and the government service area (**Figure 3**). In the icddr,b service area, four administrative blocks (called block A, B, C, and D; **Figure 3**) are connected with a central hospital. Each block has a sub-center (health care facility), where paramedical staff provides maternal and child health care, including delivery services. All sub-centers are equipped to provide public-health and clinical research support covering a population of 110,000. The studies in the present thesis (**Paper I-IV**) have been conducted in the icddr,b service area (**Figure 3**). The HDSS database is updated based on information collected through monthly home visits by community health research workers (CHRWs), who record all vital events such as deaths, marriages, in- and out-migrations, pregnancies, pregnancy outcomes, and selected child and maternal morbidity events.

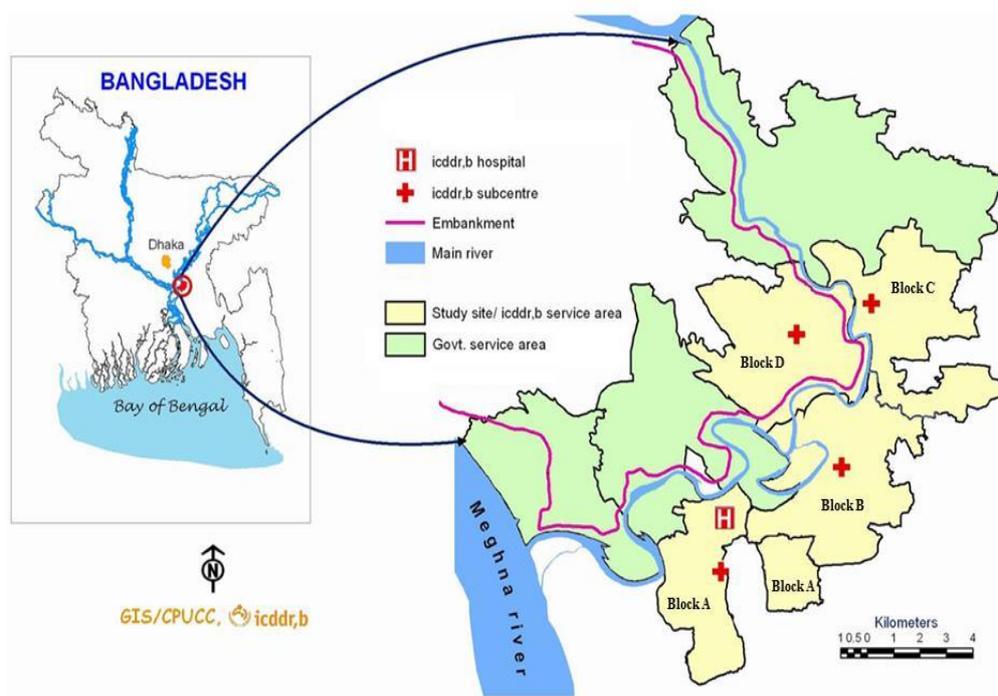


Figure 3: Map of Bangladesh and Matlab study area [adapted from (Nasreen et al. 2014) with minor modifications].

3.2 STUDY DESIGN AND PARTICIPANTS

The four papers in the present thesis are based on data from pregnant women enrolled in the MINIMat (Maternal and Infant Nutrition Interventions, Matlab) trial, and children who were born to mothers in the MINIMat trial and thereafter followed-up at 4.5 and 9 years of age. The MINIMat trial (ISRCTN16581394) recruited pregnant women from November 2001 to October 2003. Community health research workers, employed by HDSS, visited every household in the icddr,b service area on monthly basis and offered a urine-based pregnancy test (ACON, United States) to all women who reported that their menstrual period was overdue by at least two weeks. Women were invited to provide the remaining urine sample for analysis of arsenic concentration in case of a positive response on the pregnancy test. Thus, the initial urine samples were collected around gestational week (GW) 8 (median 8 weeks; mean 9 weeks, and standard deviation 3 weeks). Women with a positive pregnancy test were encouraged to visit their closest care facility, where they underwent an ultrasound examination. Women were enrolled in the MINIMat trial based on the following criteria: viable fetus by ultrasound examination, gestational age of less than 14 weeks, women had no severe illness, and gave consent to participate. The enrolled women (n=4,436) were randomly assigned to one out of two food supplementations [early food supplementation (start at around 9 weeks of pregnancy) or usual food supplementation (start around 20 weeks of pregnancy)], as well as one out of three different micronutrient supplementations (start at 14 weeks of pregnancy). The three types of micronutrient supplements were: a) 30 mg iron and 400 µg folic acid, b) 60 mg iron and 400 µg folic acid, or c) a multiple micronutrient capsule (15 micronutrients including 30 mg iron and 400 µg folic acid). An additional urine sample

was collected for measurement of urinary arsenic during late gestation at about GW30 (median 30.5 weeks; mean 30 weeks, standard deviation 2 weeks), during a visit for an antenatal check-up at a health care facility.

For the studies presented in **Paper I-II**, we selected all women in the MINIMat trial from July 2002 to September 2003 (n=300), who later on gave singleton birth in the hospital or any of the health care facilities during early day time (5:00 a.m.–2:30 p.m.). We managed to collect placental tissue and cord blood from 286 out of these 300 women. However, only 130 placenta samples were available for the analyses of immune markers (**Paper I**) and 130 cord blood samples contained enough lymphocytes for measurements of thymic function (**Paper II**). Due to the high frequency of home deliveries and delivery outside of our coverage time, the sample size was small. It was limited because of logistic difficulties of processing and transferring samples from Matlab to the laboratory in Dhaka.

MINIMat children were followed-up at 4.5 and 9 years of age (**Paper III-IV**). In total, 2,735 of the MINIMat children were followed-up at 4.5 years of age for a number of different outcomes. On the basis of month and year of birth children were divided into two groups (Group A, April 2002 to June 2003; Group B, June 2003 to June 2004). Studies related to immune function were conducted in Group B (n=1,303) between December 2007 to February 2009. The study to assess immune response in relation to arsenic exposure included 640 of those children (born June 2003 to June 2004) with available urine and blood samples (**Paper III**). We followed up the same children (n=640) at 9 years of age between June 2012 to June 2013. Since the last follow-up at 4.5 years, 34 children had migrated from the study area, and 44 children were either absent or their parents did not give consent to participate in this follow-up. Thus, in total, 562 children were included at 9 years of age (**Paper IV**).

3.3 ANTHROPOMETRY AND MORBIDITY DATA

Anthropometric measures of the women were taken at enrollment (around GW8) and three more occasions (GW14, 19 and 30). The study interviewers visited all participating women and registered pregnancy outcomes (spontaneous abortion, induced abortion, stillbirth, and live birth). When deliveries took place at health facilities, the attending nurse measured the newborn's anthropometry (weight, length, and head circumference). At home deliveries, birth anthropometry was measured by trained paramedics generally within 72 hours of birth (Persson et al. 2012).

Based on a set of structured questionnaires, health research workers collected morbidity information from pregnant women during monthly scheduled home visits. Four specific morbidity questions were included in the questionnaires concerning respiratory illness (in terms of cold, cough, or difficult breathing), diarrhea/dysentery (three or more liquid stools in 24 hours or stools mixed with blood), and urinary tract infections (in terms of burning/pain/difficulty during urination) with or without concomitant fever. The duration of the illness (number of days) was also recorded.

At 4.5 and 9 years of age, children's height and weight were recorded using a standard procedure. The measured height and weight were converted to height-for-age and weight-for-age Z-scores (SD scores), using the WHO growth reference for preschool and school-aged children (De Onis 2006; De Onis et al. 2007). Information on child morbidity at 4.5 years (two weeks recall) of age was collected by the study interviewers at home visits. The questionnaire included specific morbidity questions concerning acute respiratory illness and diarrhea.

3.4 SOCIOECONOMIC STATUS (SES)

Socioeconomic status (SES) of the families was retrieved from the HDSS databases at the enrollment in the MINIMat trial (**Paper I-II**). Family SES was also updated during the follow-up of the children at 4.5 years (**Paper III**) and 9 years (**Paper IV**) of age. SES was defined in terms of assets on the basis of household ownership of different items (e.g., fan, bicycle, mobile phone, or television), dwelling characteristics (e.g., flooring and roofing material and number of people per room), type of household sanitation, type of cooking fuel, and drinking-water sources used. SES score was generated through principal component analysis by assigning a weight or factor for each household asset (Gwatkin et al. 2000). Household asset scores were divided into quintiles (1 representing the poorest and 5 the richest). All women were nonsmokers and did not ingest alcoholic beverages; however, 10-20% reported tobacco chewing. The "chewing tobacco" comprised of dried tobacco leaves locally known as zarda (sweetened tobacco leaves) or gul (dried and powdered tobacco). Zarda is taken in a mixture of a leaf of the piper betel plant, together with sliced areca nut and lime, whereas gul is placed between the gums to be sucked slowly (Lindberg et al. 2010).

3.5 EXPOSURE ASSESSMENTS

3.5.1 Urinary arsenic

Exposure to inorganic arsenic was assessed by the concentration of inorganic arsenic (As^{III}, As^V) and its methylated metabolites (MMA, DMA) in urine collected from the pregnant women at GW8, 14 (in subsequent pregnancies), and 30, and from the children at 4.5 and 9 years of age. Urine samples were collected into a disposable metal-free plastic cup (Papyrus, Gothenburg, Sweden) from which urine was transferred to a 24-mL polyethylene bottle tested essentially free from trace elements (Zinsser Analytic GMBH, Frankfurt, Germany). In early pregnancy, spot urine samples were collected in the women's home whereas the collection of spot urine in late pregnancy and from the children at 4.5 and 9 years of age occurred at the health care facilities. After collection, the samples were chilled and kept cold until frozen at the end of the day at the latest. Later, the frozen urine samples were transported to the laboratory in Karolinska Institutet, Sweden, for measurements of urinary arsenic (U-As). Maternal urinary arsenic was assessed by hydride generation-atomic absorption spectroscopy (HG-AAS) (Vahter et al. 2006) (**Paper I- II**). Children's urinary arsenic at 4.5 (**Paper III**) and 9 years (**Paper IV**) of age, and their mothers' urinary arsenic (**Paper III-IV**) in early pregnancy were measured by high-performance liquid chromatography online with hydride

generation and inductively coupled plasma mass spectrometry (HPLC-HG-ICPMS) as described previously (Gardner et al. 2011). For quality control purposes, we used reference materials when we measured urinary arsenic using HG-AAS and HPLC-HG-ICPMS, which are described in detail in all respective papers (**Paper I-IV**).

In order to compensate for variation in dilution in urine samples urinary arsenic concentrations were adjusted to the specific gravity of the urine, measured by a digital refractometer (RD712 Clinical Refractometer; EUROMEX, Arnhem, the Netherlands). The difference in dilution of urine is caused by variation in fluid intake as well as differences in physical activity and temperature (Nermell et al., 2008). The average specific gravity of both maternal urine samples and the children's urine samples at 4.5 and 9 years of age was 1.012. Adjustment by specific gravity has been found to be less affected by age, body size, gender, and season, compared to the more commonly used creatinine adjustment (Nermell et al. 2008).

3.5.2 Blood and placenta arsenic

Total arsenic in maternal erythrocytes, cord blood erythrocytes, and placental tissue was measured by inductively coupled plasma mass spectrometry (Agilent 7500ce, Agilent Technologies, Tokyo, Japan) (Kippler et al. 2010) after digestion of the samples, using a Milestone UltraCLAVE II microwave digestion system (EMLS, Leutkirch, Germany). A detailed description of the methods is given in **Paper II**.

3.6 OUTCOMES

The following section provides an overview and evaluation of the methodology used to measure the different outcomes in the present thesis. The detailed description of a particular method/assay can be found in the referenced papers (**Paper I-IV**).

3.6.1 Oxidative stress, immune cells, and cytokines in placenta

Immunohistochemical methods were used for the quantitative detection of immune cells expressing the markers CD3 (pan T cell), CD8 (cytotoxic T cells), CD64 (FcγRI, monocytes/macrophages), and CD68 (phagocytes); myeloperoxidase (MPO); the cytokines interleukin-1 β (IL-1 β), interferon- γ (IFN γ), leptin ob (Y-20), tumor necrosis factor- α (TNF α), IL-6, and IL-10; and 8-oxoguanine (8-oxoG) (**Paper I**).

3.6.2 Thymic function in newborns

During T cell selection in the thymus, Signal joint T cell receptor excision circles (sjTRECs) are formed as a by-product of T cell receptor gene rearrangements. Thymic function of the newborns was assessed by measuring sjTRECs. Real time polymerase chain reaction (RT-PCR) was used to measure sjTRECs in cord blood mononuclear cells (CBMC) and in separated CD4⁺ and CD8⁺ cells using specific primers (**Paper II**).

3.6.3 Gene expression in cord blood

We measured 84 pre-defined genes involved in oxidative stress and the antioxidant defense system, and 84 genes involved in the apoptosis pathways (SABiosciences) in six newborns with the highest arsenic concentrations in cord blood (mean \pm SD, 19.3 ± 11.4 $\mu\text{g}/\text{kg}$) and six newborns with the lowest arsenic concentrations in cord blood (1.1 ± 1.0 $\mu\text{g}/\text{kg}$) for comparison of gene expression in CBMC. Real-time PCR array was carried out according to the manufacturer's instruction (SABiosciences) (**Paper II**).

3.6.4 8-OHdG in cord blood

Plasma concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in cord blood were analyzed by a competitive ELISA kit (Highly Sensitive 8-OHdG Check ELISA, JaICA, Japan) according to the manufacturer's instruction. Before the analysis, the samples were filtered (Amicon Ultra-0.5, Millipore, Billerica, MA) by centrifugation at 14,000 rpm to remove the proteins with molecular weight > 10 kDa, which could interfere with the measurements of 8-OHdG (**Paper II**).

3.6.5 Plasma cytokines

We measured plasma cytokines using a Bio-Plex Pro Human Cytokine 18-Plex Panel in cord blood plasma (**Paper I**) and Bio-Plex Pro Human Cytokine 27-Plex Panel (**Paper III**) in children's blood plasma at 4.5 years of age using a Bio-Plex 200 reader (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

3.6.6 Cell-mediated immunity

Children's cell-mediated immunity was measured by measuring delayed type hypersensitivity in response to purified protein derivative (PPD) (**Paper III**). The children were given an intradermal injection of PPD into the middle part of volar surface of the left forearm and the induration size was measured after 48-72 hours.

3.6.7 Vaccination-specific IgG concentrations

Enzyme immunoassays were used for the quantitative determination of Measles-, Mums-, and Rubella-specific IgGs in children at 9 years of age according to the manufacturer's instructions (IMMUNOLAB GmbH, Kassel, Germany) (**Paper IV**).

3.6.8 Total plasma IgG concentrations

Immunoturbidimetric assay was used for the quantitative determination of plasma total IgG in children at 9 years of age (Roche Diagnostic GmbH, D-68298 Mannheim, Germany) according to the manufacturer's recommendations (**Paper IV**).

3.7 ETHICAL CONSIDERATION

Both oral and written informed consent was obtained from the pregnant women about their participation in the MINIMat trial. Written informed consent was obtained from the mother

or legal guardian of each child prior to participation in the present studies. Enrolled mothers and their children were informed that they could refrain from the study at any time point without affecting their access to routine health services. The studies have been approved by the Research Review Committee and Ethical Review Committee at icddr,b, Bangladesh and the Regional Ethical Committee at Karolinska Institutet, Sweden.

3.8 STATISTICAL ANALYSES

Statistical analyses were conducted using the software PASW 22.0 (SPSS Inc. Chicago, USA) and Stata/IC, version 12.1 (StataCorp, Texas, USA). Associations between exposures, outcomes, and covariates were first evaluated using Spearman's rank correlation coefficient (for continuous variables), Mann-Whitney U test, analysis of variance, or Kruskal-Wallis test (for categorical variables), or other non-parametric test as appropriate.

Effects of arsenic exposure on outcomes were evaluated by multi-variable adjusted models (linear regression, analysis of covariance, and log-binomial regression). If the associations between exposure and outcome were not linear, we categorized the exposure and the lowest category was used as the reference. When necessary, exposure and outcome variables were \ln (natural logarithm) transformed to obtain normally distributed residuals with a homogeneous variance. The statistical models were adjusted for covariates that were associated with exposure and outcomes, and if the covariates changed the effect estimates by 5% or more.

4 RESULTS AND DISCUSSION

This section summarizes the main results, including some unpublished data along with discussion. For further details about the results and discussion, the reader is referred to the individual papers (**Paper I-IV**).

4.1 EXPOSURE ASSESSMENTS

Urinary arsenic concentrations were used as a biomarker of arsenic exposure. Arsenic is absorbed by the gastrointestinal tract and excreted in urine within a few days. If individuals are exposed daily through water and food, it is likely that the excretion of arsenic and its metabolites through urine reach a steady state level. Therefore, urinary arsenic concentration reflects ongoing exposure to inorganic arsenic from all sources, i.e. both drinking water and food (Vahter et al. 2006). In **Paper I-IV**, we assessed arsenic exposure by the sum concentration of iAs and its methylated metabolites (MMA and DMA) in urine, hereafter referred to as U-As. In children, we also measured total arsenic in urine by ICPMS. We found an excellent agreement between sum metabolites concentrations measured by HPLC-HG-ICPMS and total arsenic concentrations measured by ICPMS ($R^2=0.993$). This indicates that the intake of seafood is minimal in the study area. In **Paper II**, we also used blood arsenic and placental arsenic along with U-As as exposure biomarkers. Sometimes hair and nail samples are used as long-term biomarkers of inorganic arsenic exposure occurring over the past several months (Hughes 2006). However, analysis of hair and nails were not considered in this highly exposed population because of the risk of external contamination (washing and bathing in contaminated water).

Since 2001, considerable mitigation efforts have been undertaken in the study area. People have been encouraged through community education to switch to tube-wells with low arsenic concentration ($<50 \mu\text{g/L}$), which were painted green. Other mitigation options, for example, community-based arsenic removal filters and rainwater harvesting, were also promoted (Jakariya et al. 2007). A review reported that many people who had access to contaminated well water ($>50 \mu\text{g/L}$) now changed their water source to a new or existing lower arsenic ($<50 \mu\text{g/L}$) water source (Johnston and Sarker 2007).

The median U-As concentration was $79 \mu\text{g/L}$ (range $2\text{-}2,064 \mu\text{g/L}$) in maternal urine at GW8, $57 \mu\text{g/L}$ (range $12\text{-}1,228 \mu\text{g/L}$) in child urine at 4.5 years, and $53 \mu\text{g/L}$ (range $9\text{-}1,268 \mu\text{g/L}$) in child urine at 9 years of age (Table 1 and **Figure 4**). Children's U-As at 9 years of age was significantly correlated with their U-As at 4.5 years of age ($r_s=0.56$, $p<0.001$, **Figure 5A**) and with maternal U-As during early pregnancy ($r_s=0.36$, $p<0.001$, **Figure 5B**). Children's U-As at 4.5 years of age was associated with maternal U-As during early pregnancy ($r_s=0.45$, $p<0.001$, **Figure 5C**). There was a significant reduction of U-As in children at 9 years of age compared to that of their mothers (Wilcoxon signed-rank test, $p<0.001$), although the concentrations were correlated. However, there seemed to be no change in U-As concentrations since 4.5 years of age (**Table 1** and **Figure 4**). In total 242 mothers had high U-As ($\text{U-As}>100 \mu\text{g/L}$) at GW8, 40% of their children also had very high

U-As at 9 years of age. Of the 194 mothers with U-As < 50 µg/L, 65% of their children had U-As < 50 µg/L at 9 years of age. Only 14% of the children at 9 years of age had U-As values > 50 µg/L, which was higher than their mothers' at GW8. Gardner et al. also reported similar concentrations of U-As in a larger sample size (n=1951) in children at 5 years of age (Gardner et al. 2011) in the same study area, with a median of 51 µg/L (range 4-1,496 µg/L) (Gardner et al. 2011). Similar or higher concentrations of U-As in children (4-10 years) were reported in other regions in Bangladesh (Hall et al. 2009; Nahar et al. 2014). In contrary, the U-As concentrations of our studied children were 8-10 times higher than in children of a similar age in Germany, the United States, and Taiwan (Chiang et al. 2008; Caldwell et al. 2009; Schulz et al. 2009). Our observed decrease of median U-As concentrations over time (10 years) ($p < 0.001$, sign rank test) is probably due to the extensive arsenic remediation efforts. However, examination of the U-As distribution at each time point (**Figure 5**) demonstrated little change over time. The exposure levels are still highly elevated.

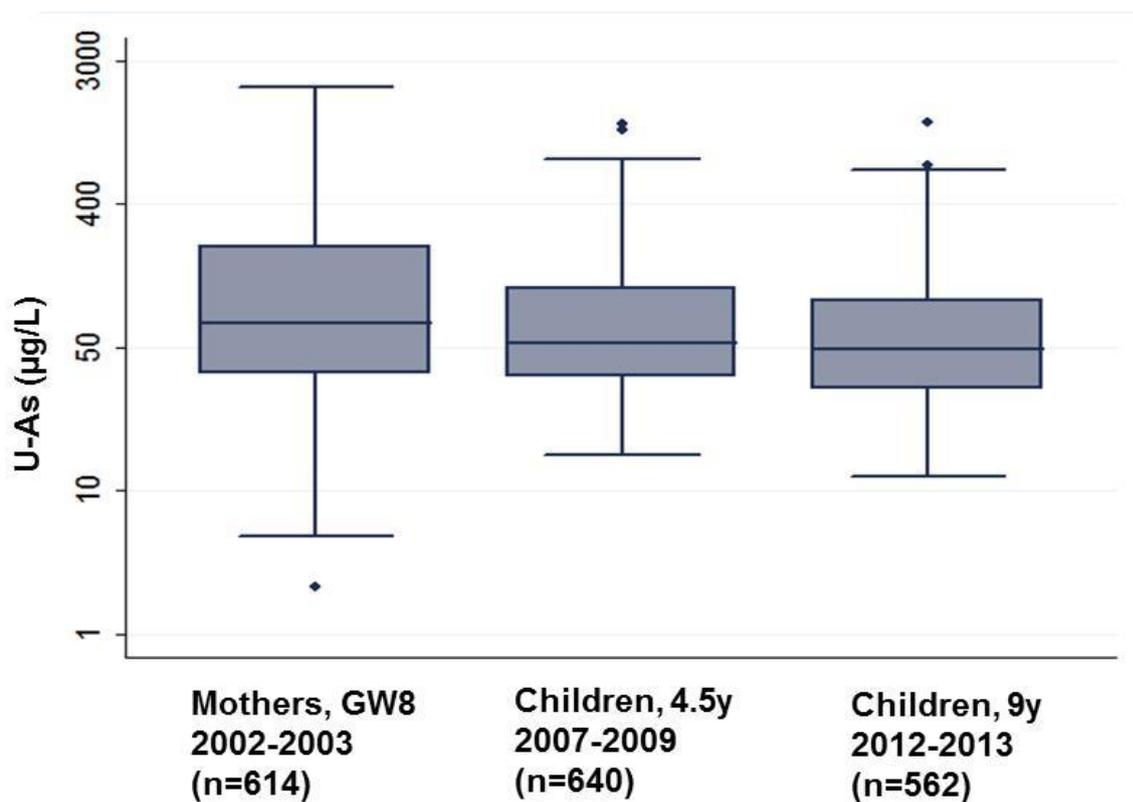


Figure 4: Urinary arsenic concentrations in maternal urine at GW8, and children's urine at 4.5 and 9 years of age.

Table 1: Arsenic concentrations in maternal urine at GW8, and children’s urine at 4.5 and 9 years of age: Matlab, 2002-2013.

	U-As ($\mu\text{g/L}$), GW8	U-As ($\mu\text{g/L}$), 4.5 years	U-As ($\mu\text{g/L}$), 9 years
Median	79	57	53
Mean	187	111	98
Minimum	2	12	9
10 th percentile	26	25	22
90 th percentile	494	267	242
Maximum	2064	1228	1268
N	614	640	562

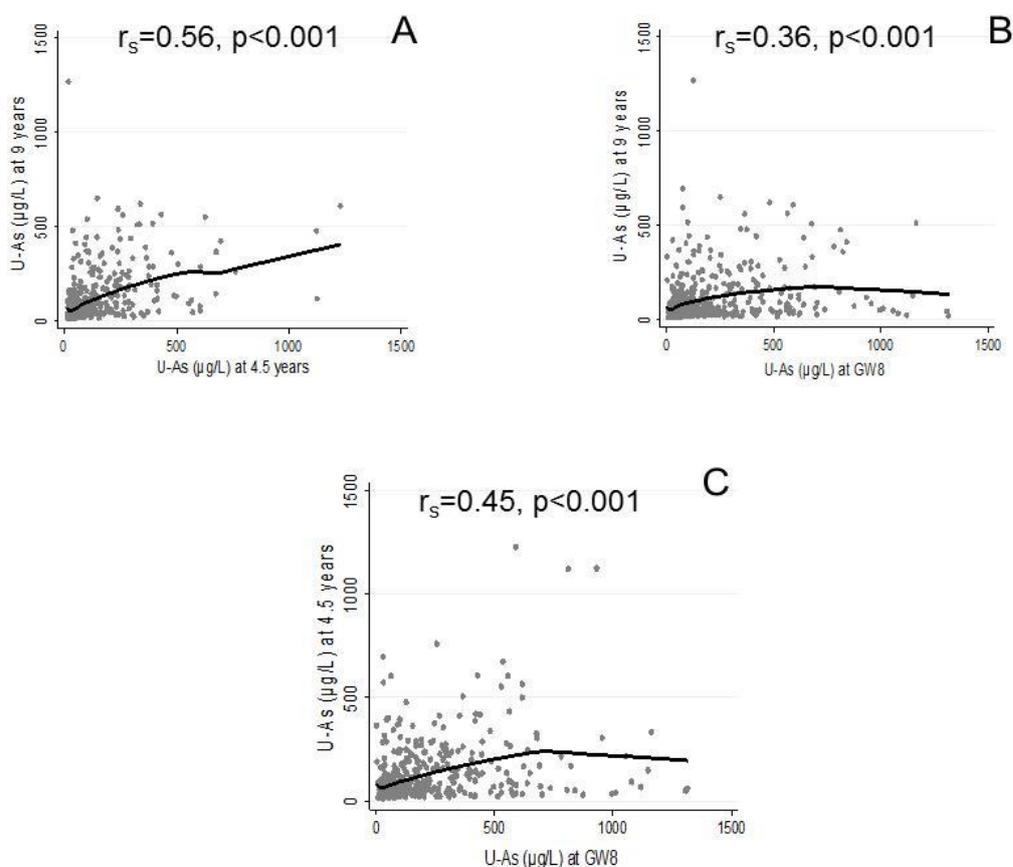


Figure 5: Association of urinary arsenic concentrations between (A) children at 9 years and 4.5 years, (B) children at 9 years and their mothers at GW8, and (C) children at 4.5 years and their mothers at GW8. Solid line indicates lowest line.

In the present studies (**Paper I-IV**), U-As levels were related to geography, socioeconomic status, and mother’s education. The highest U-As concentrations were found in block C and the lowest concentrations in block D. This geographical variation is probably related to local variations in arsenic concentrations in tube-well water in Matlab, as found in other parts of Bangladesh (Kinniburgh and Smedley 2001). Mother’s education and SES were inversely associated with U-As, indicating that people with higher SES have started shifting to water sources to those with less arsenic. In a sub-sample (n=208), we found a good

agreement between water arsenic and U-As at 9 years of age ($r_s=0.78$, $p<0.001$). However, the median water arsenic concentration was very low ($4.7 \mu\text{g/L}$, range $0.05\text{-}743 \mu\text{g/L}$) which indicates other sources of arsenic exposure, such as food. High concentrations of iAs have been found in rice, which is the main staple food in Bangladesh (Gardner et al. 2011; Meharg et al. 2014). Rice may be contaminated through the use of arsenic contaminated cooking water or by the uptake of arsenic by the rice plant from soil contaminated due to the use of well water for irrigation purposes. For approximately 10% ($n=57$) of the children at 9 years, an additional urine sample was collected 21 days after the first urine collection. We found a good agreement between these two measures ($r_s=0.86$, $p<0.001$), indicating a steady state level of excretion of arsenic in urine over time due to continuous exposure.

4.2 ARSENIC EXPOSURE AND IMMUNE CELLS IN PLACENTA

Maternal U-As in early pregnancy (GW8) was inversely associated with the frequency of placental CD3+cells (total T cells) in a dose dependent manner (**Figure 6**). The number of placental CD8+ cells also decreased with increasing maternal U-As, but the association was not statistically significant (**Paper I**).

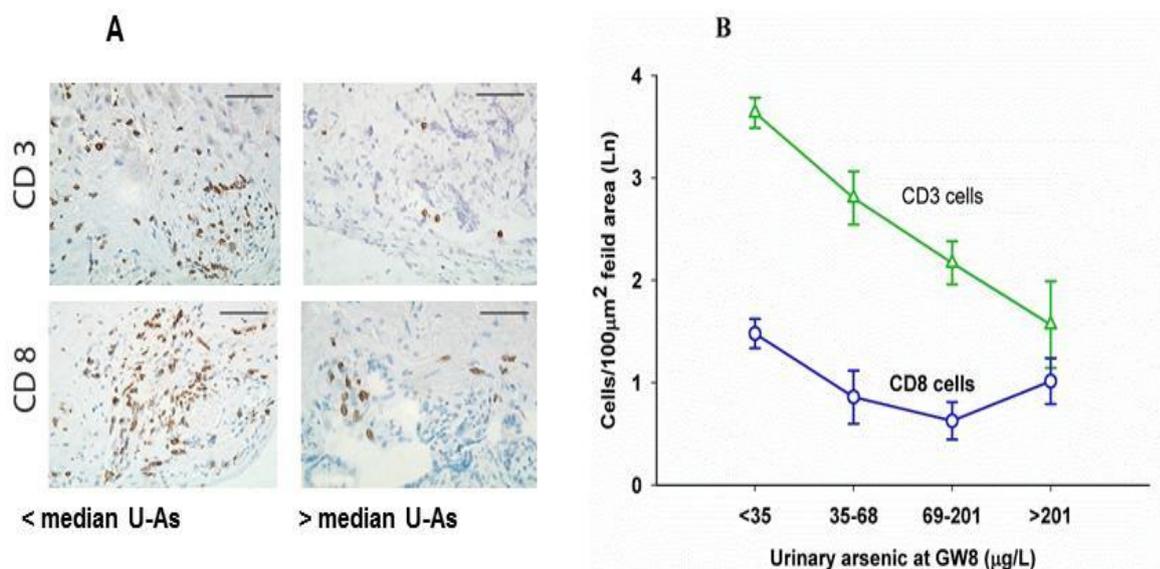


Figure 6: (A) Number of placental T cells in relation to maternal arsenic exposure. (B) Association between maternal arsenic exposure in quartiles and the frequency of placental T cells.

Our results suggest that maternal arsenic exposure during pregnancy inhibits placental total T cells, and as a consequence there may alter placental immunity. To our knowledge, this is the first report examining placental immunity in relation to maternal arsenic exposure. Our results are in line with several studies of T cell counts and functions in children and adults. A recent study conducted in Bangladesh showed an inverse association between maternal arsenic exposure via drinking water (median $12 \mu\text{g/L}$, range $< 1\text{-}510 \mu\text{g/L}$) during pregnancy and cord blood CD4+ cells (Kile et al. 2014). Arsenic exposure in Mexican children (6 to 10 years old) showed reduced percentage of CD4+ T cells with increasing

concurrent U-As; however data was missing regarding their prenatal exposure (Soto-Pena et al. 2006). Another study in seemingly healthy adults with long-term arsenic exposure showed that U-As concentrations were significantly inversely associated with the number and function of natural T regulatory lymphocytes (Hernandez-Castro et al. 2009). These T regulatory cells are important in maintaining immune tolerance and immune homeostasis in the maternal-fetal interface (Miyara and Sakaguchi 2007; Sharma 2014). One key feature of T regulatory cells during pregnancy is that they increase their numbers in the maternal-fetal interface and release the immunosuppressive cytokines IL-10 and TGF- β , which are involved in maintaining placental immune tolerance (Sharma 2014). In our study, we did not measure placental T regulatory cells; however, it is possible that arsenic specifically affects T regulatory cells among total T cells.

4.3 ARSENIC EXPOSURE AND THYMIC FUNCTION IN NEWBORNS

The thymus is the primary site for T cell generation and selection, particularly during the fetal period and early childhood. Newborn thymic function (number of naïve T cells) was measured by the quantification of Signal-joint T-cell receptor-rearrangement excision circles (sjTREC_s) in cord blood mononuclear cells, and also in separated CD4⁺ and CD8⁺ cells (**Paper II**). We found an inverse nonlinear relationship between maternal arsenic exposure and cord blood sjTREC_s (**Figure 7A**). Similar results were also observed in separated CD4⁺ and CD8⁺ cells (**Figure 7B**). High levels of sjTREC_s (above the median) in women with low arsenic exposure at GW30 (<50 $\mu\text{g/L}$) were positively associated with maternal morbidity during pregnancy ($r_s=0.33$, $p=0.09$). On the other hand, maternal morbidity was positively associated with maternal arsenic exposure during pregnancy ($r_s=0.20$, $p=0.01$). Thus, the main finding was the absence of high sjTREC_s at high arsenic exposure levels, despite the apparent arsenic-associated maternal morbidity.

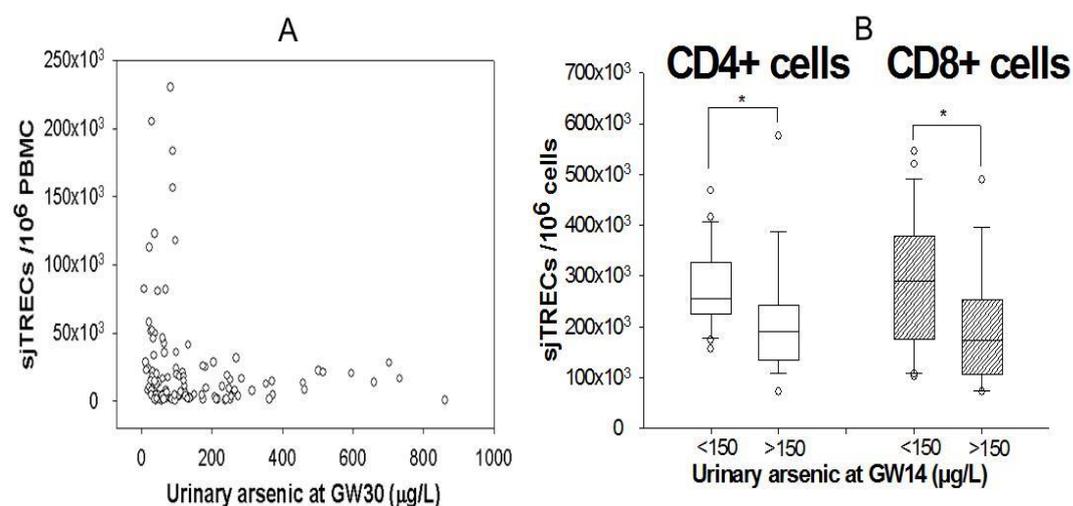


Figure 7: The associations between maternal urinary arsenic during pregnancy and sjTREC_s in (A) cord blood mononuclear cells, and (B) separated CD4⁺ and CD8⁺ cells.

These results indicate that the production of naïve T cells in the fetal thymus may be inhibited by maternal arsenic exposure during pregnancy, particularly affecting the

response to maternal infection. The arsenic-exposed neonatal immune system appears to be unable to mount a proper immune response to infections (by producing enough T cells). This could be because of the state of anergy of the thymocytes, resulting in inability to produce required T cells. Another reason could be arsenic-induced apoptosis of fetal thymocytes or naïve T cells. Indeed, we observed that some apoptosis-related genes, including CASP2 and CASP8, were up-regulated in cord blood mononuclear cells in relation to maternal arsenic exposure (**Paper II**). A recent study has reported an inverse association between maternal arsenic exposure during pregnancy and cord blood naïve T cell (CD45RA+ CD4+ CD69+) counts (Nadeau et al. 2014). Previously we observed that maternal arsenic exposure reduced fetal thymus size (Moore et al. 2009; Raqib et al. 2009). Studies in West Africa have shown that infants with a smaller thymus had an increased risk of childhood infection-related mortality (Aaby et al. 2002; Garly et al. 2008). Similar results were also observed within a larger sample of the MINIMat children (n=1,715) where thymic index at 8 weeks of age was inversely associated with subsequent infection-related mortality up to 5 years of age (Moore et al. 2014). Thus, the reduced thymic size and function of the infants, induced by the maternal arsenic exposure, will likely render them more susceptible to infections.

4.4 ARSENIC EXPOSURE AND CELL-MEDIATED IMMUNE FUNCTION IN CHILDREN AT 4.5 YEARS OF AGE

Based on the indicated arsenic related immunotoxicity (reduced T cells in placenta and reduced naïve T cells in cord blood) in newborns (**Paper I and II**), we followed the immune status of the children at 4.5 years of age. We evaluated cell-mediated immune function by measuring delayed type hypersensitivity in response to PPD injection in children vaccinated against Bacillus Calmette-Guerin (**Paper III**). Children's arsenic exposure was positively associated with the risk of not responding to the PPD (induration <5mm), with some evidence for a similar trend with children's prenatal exposure (**Figure 8**). Higher risk of not responding to PPD was observed in children in the highest quartile of U-As (range 126-1,228 µg/L), compared with those in the lowest quartile (range 12-34 µg/L) [RR=1.37 (95% confidence interval (CI): 1.07, 1.74)]. Further analysis revealed that the association was stronger in children with recent infections (RR=1.55, 95% CI: 1.04, 2.32) (**Paper III**).

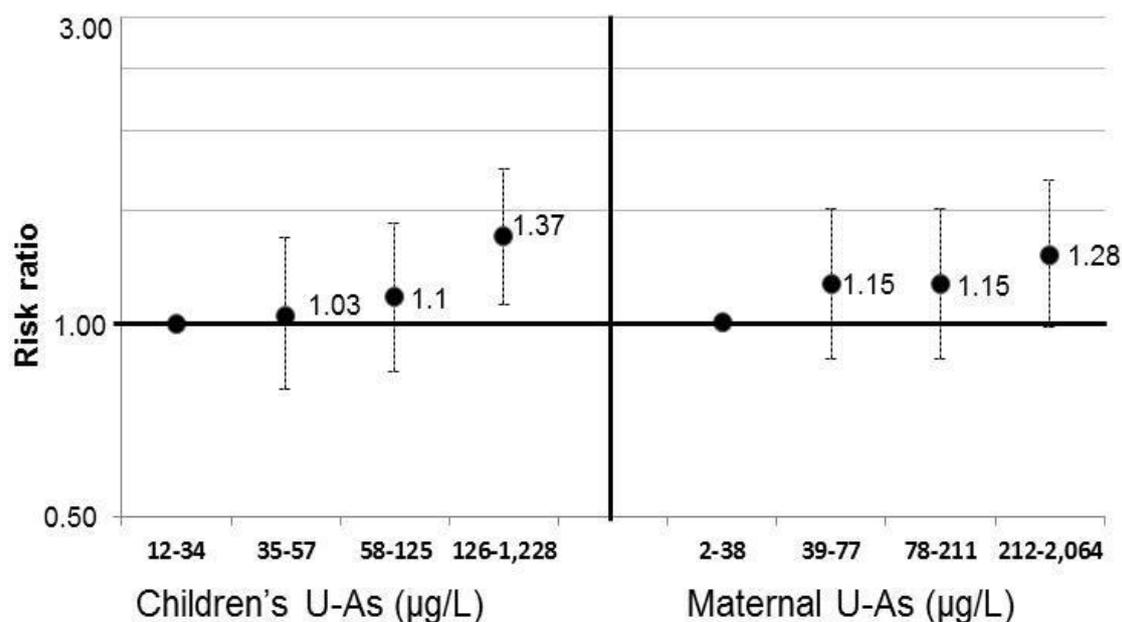


Figure 8: The risk of not responding to the injected PPD in children at 4.5 years of age in relation to concurrent and prenatal arsenic exposure in quartiles.

For the assessment of developmental immunotoxicity, the delayed type hypersensitivity test is a well-accepted method used as a functional measure of an individual's cell-mediated immunity (Descotes 2006; Siripassorn et al. 2006; Vukmanovic-Stejic et al. 2006; Dietert et al. 2010). Macrophages and T cells are mainly involved in cell-mediated immunity (Abbas et al. 2012). The observed decreased cell-mediated immune function in relation to children's arsenic exposure again indicates arsenic-related immunosuppression. This is probably due to arsenic-induced reduction of T cells (Soto-Pena et al. 2006; Kile et al. 2014) or impairment of T cell function, in particular of CD4+ helper T cells. It could also be due to arsenic-related reduction of macrophages or impaired macrophage function (Dangleben et al. 2013). Indeed, impaired macrophage function (e.g. reduced adhesion capacity, phagocytic function, and expression of F-actin) was observed in arsenic-exposed adults with skin lesions compared to unexposed individuals (Banerjee et al. 2009). Our observed arsenic-related effects on cell-mediated immunity in children with recent infections indicate that arsenic impairs the immune response to infections. Thus, arsenic exposure may render the children more susceptible to infections.

4.5 ARSENIC EXPOSURE AND PLASMA TOTAL IGG IN CHILDREN AT 9 YEARS OF AGE

While we have seen that arsenic affects T cells and T cell-associated function (**Paper I-III**), we have also evaluated the humoral immune function in children (n=534) at 9 years of age (**Paper IV**). Children's total plasma IgG (tIgG) concentrations were measured using immunoturbidimetric assay. We observed significantly higher concentrations of tIgG in children in the highest tertile of maternal U-As compared with the children in the lowest tertile (**Figure 9**; p for trend =0.027). When we stratified the analysis by gender, the effect was only evident in boys (p for trend =0.005). Increasing concentrations of plasma tIgG

were also observed with increasing child U-As at 4.5 and 9 years of age, but the associations were not statistically significant (**Paper IV**).

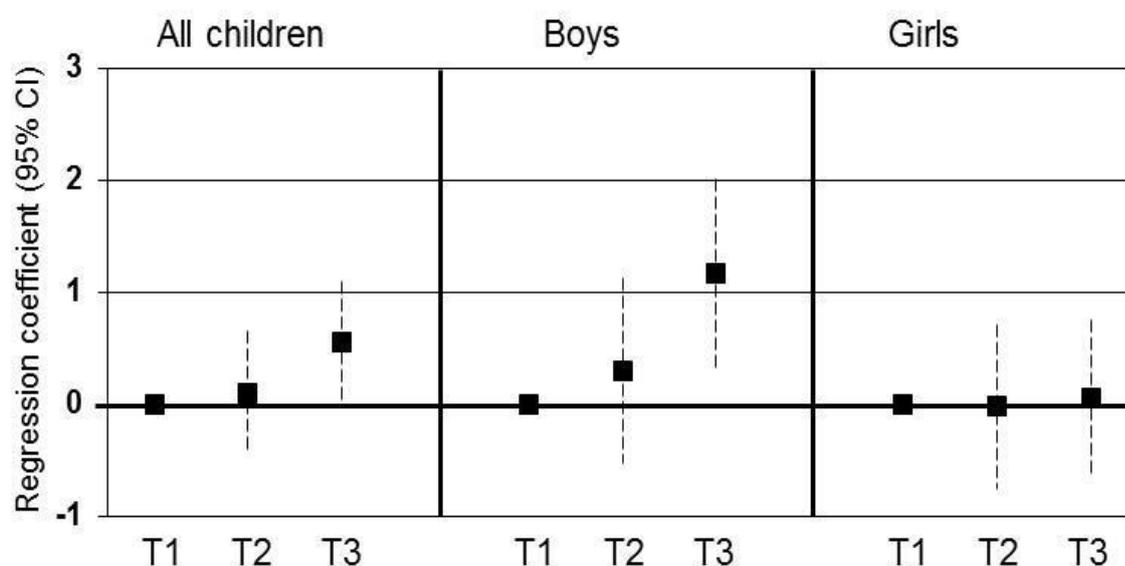


Figure 9: Maternal urinary arsenic in tertiles and plasma total IgG in children at 9 years of age. T1, 2-47 $\mu\text{g/L}$; T2, 48-170 $\mu\text{g/L}$; and T3, 171-2,064 $\mu\text{g/L}$.

IgG is the main immunoglobulin in plasma, synthesized by B cells. It is involved in neutralizing pathogens and their toxins in the circulation and tissue fluids (Abbas et al. 2012). However, elevated concentrations of serum IgG have also been associated with increased risk of infections and autoimmunity in children (Lo et al. 2013). We observed the strongest association in relation to maternal arsenic exposure, irrespective of subsequent childhood exposures, indicating that the maternal arsenic exposure during pregnancy altered fetal B cell programming. A recent study showed that prenatal arsenic exposure altered the expression of microRNAs in cord blood which were associated with reduced expression of immune-related genes, including B cell receptor signaling genes (Rager et al. 2014). Another explanation for the increased plasma total IgG in relation to maternal arsenic exposure could be arsenic-induced imbalance between Th1 and Th2 cytokines in children (lower Th1 cytokines than normal), which might result in up-regulation of the nonspecific antibody production (Kidd 2003). It could also be due to the compensatory mechanism of arsenic-induced reduction of T cells. Possibly that in a state of T cell paucity or impaired T cell function, the immune system may try to compensate the void of T cells by producing excessive levels of nonspecific antibodies. For instance, HIV-infected children were shown to have higher levels of plasma total IgG in presence of very low number of CD4+ helper T cells in the early stages of infection (Shearer et al. 2000).

The effect of early life arsenic exposure on plasma IgG was only obvious in boys, indicating that boys are more susceptible than girls to certain immune-related health effects, such as infections. This result contributes to the growing evidence that at least some of the effects of arsenic are gender-specific (Vahter 2009; Farzan et al. 2013). Indeed, stronger

effects of maternal arsenic exposure during pregnancy on acute respiratory infections were indicated in male infants than in female in a sub-sample (n=140) of the cohort (Raqib et al. 2009). Similarly, prenatal arsenic exposure has been associated with reduced fetal growth and genome-wide DNA hypomethylation in cord blood in boys with limited evidence in girls (Kippler et al. 2012; Broberg et al. 2014).

4.6 ARSENIC EXPOSURE AND MMR VACCINATION-SPECIFIC PLASMA IGG IN CHILDREN AT 9 YEARS OF AGE

We measured vaccination-specific IgG antibodies in children, as it reflects a major immune system functional endpoint. Unfortunately, we do not have a sero-negative group for measles, mumps and rubella antigens (with pre-vaccination titers <10 U/mL), indicating some prior exposure to these viruses in all children. The increase in post-vaccination titers is dependent on pre-vaccination titers. Therefore, we evaluated post-vaccination IgG titers in relation to arsenic exposure in different quartiles of pre-vaccination IgG titers (**Paper IV**). In the lowest quartile of pre-vaccination IgG titers, children's U-As at 4.5 and 9 years of age, but not maternal U-As, were significantly inversely associated with mumps-specific post-vaccination plasma IgG [regression coefficient and (95% CI): -0.18 (-0.35, -0.01) and -0.16 (-0.32, -0.01) for U-As at 4.5 and 9 years, respectively].

The indicated arsenic-induced reduction of mumps-specific post-vaccination IgG concentrations suggests either arsenic-induced reduction of specific B cells or impaired function of specific T cells, as T cells activate B cells to secrete antigen-specific antibodies. However, there is not enough evidence of arsenic-induced reduction of B cells. Therefore, it could be speculated that arsenic-induced elimination of T helper cells or impairment of existing T cell function may lead to impaired development of antigen-specific humoral immunity. The immunosuppressive effects of arsenic observed starting in early childhood could be linked to arsenic-induced impaired antigen-specific T cell development. In contrast to our results, high levels of diphtheria and tetanus, but not measles, vaccination-specific IgG were observed in arsenic-exposed Bangladeshi children (concurrent exposure, mean urinary arsenic, 291 µg/L, n=40) compared to unexposed children (mean urinary arsenic, 7 µg/L, n=20) (Saha et al. 2013). We did not observe any significant difference of measles and rubella-specific post-vaccination IgG in relation to arsenic exposure. It is possible that arsenic exposure may affect different bacterial and viral antigen-specific IgG levels differently. Further studies are required to better understand how arsenic affects antigen-specific IgG titers.

4.7 MECHANISMS

4.7.1 Arsenic exposure and oxidative stress in placenta and cord blood

One of the main mechanisms of arsenic toxicity is oxidative stress, induced by reactive oxygen species (ROS), possibly in combination with impaired antioxidant defense (Wu et al. 2001; Vahter 2009). The use of 8-oxoguanine (8-oxoG) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) as markers of oxidative DNA damage has been reported in

several studies (Nakae et al. 2005; Engstrom et al. 2010; Flora 2011). We found that maternal U-As during pregnancy was positively associated with placental 8-oxoG (**Figure 10A and B**), already at fairly low arsenic exposure levels (<50 µg/L of U-As, **Figure 10B**) (**Paper I**). There was a clear dose response relationship between maternal U-As and placental expression of 8-oxoG (**Figure 10B**). In addition, all biomarkers of arsenic exposure (urine, placenta, and cord blood) were positively associated with cord blood plasma 8-OHdG (**Paper II**).

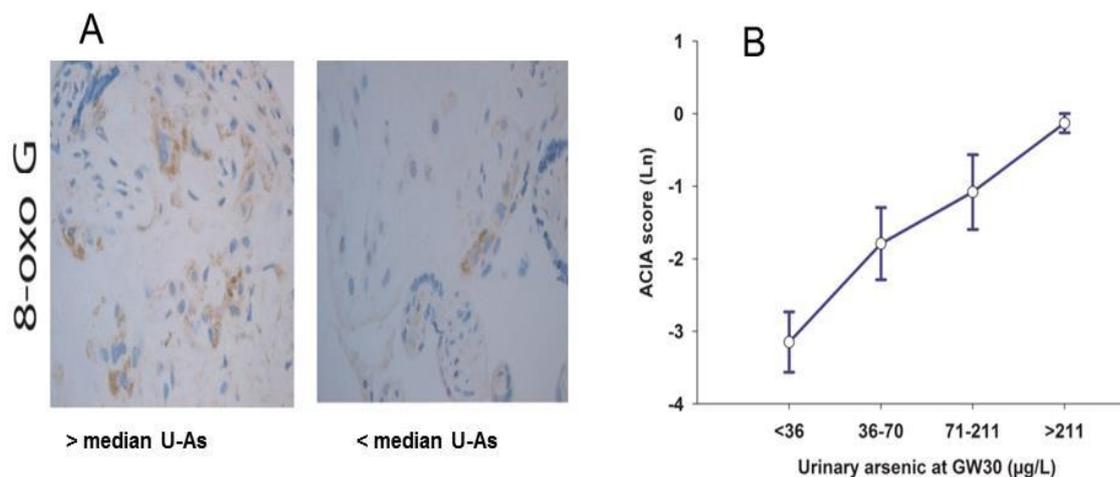


Figure 10: (A) Expression of placental 8-oxoG in relation to maternal arsenic exposure (B) Maternal urinary arsenic in quartiles and placental expression of 8-oxoG.

Arsenic-induced ROS are associated with oxidative DNA damage and lipid peroxidation, even at fairly low exposure levels (De Vizcaya-Ruiz et al. 2009; Jomova et al. 2011). We have previously shown that women in early gestation have an arsenic-related increase in urinary 8-OHdG (Engstrom et al. 2010). In agreement with these results, a study in Cambodia showed that U-As was positively associated with urinary 8-OHdG in chronically exposed individuals (Kubota et al. 2006). Probably, arsenic induces morphological changes in mitochondrial integrity and decreases mitochondrial membrane potential, resulting in increased production of ROS (Jomova et al. 2011). ROS can also be generated during the conversion of arsenite to arsenate (Rossman 2003). Superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet OH$) are the major arsenic-induced ROS. Purine and pyrimidine bases in DNA are sensitive to ROS because of the presence of unsaturated bonds, and this could lead to oxidative DNA damage. ROS production by activated immune cells is an important innate immune response to kill the pathogens. However, arsenic induced overproduction of ROS could damage immune cells as well as surrounding tissues.

Arsenic can also disrupt cellular defense mechanisms in different ways. For instance, arsenic decreases cellular glutathione levels either by utilizing it as an electron donor for the conversion of pentavalent to trivalent arsenic compounds or by oxidizing glutathione via arsenic-induced free radicals (Flora 2011). Arsenic also interferes with the DNA repair machinery, as it inhibits the expression of DNA repair enzymes (De Vizcaya-Ruiz et al. 2009). Indeed, reduced expression of nucleotide excision repair (ERCC1) enzymes in

lymphocytes was observed in an arsenic-exposed population in USA ($>6 \mu\text{g/L}$ in drinking water) compared with controls ($<5 \mu\text{g/L}$ in drinking water) (Andrew et al. 2006).

4.7.2 Arsenic exposure and gene expression in cord blood

Maternal arsenic exposure during pregnancy may alter gene expression in cord blood. Indeed, we found that several genes, involved in oxidative stress defense pathways, including superoxide dismutase (SOD), glutathione peroxidases (GPX5) and nitric oxide synthase (NOS2A) were down-regulated in cord blood mononuclear cells in relation to elevated arsenic exposure (**Figure 11**). Several studies have reported arsenic-induced depletion of antioxidant defense enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) as reviewed by Flora S (Flora 2011). We also observed that some apoptosis related genes, including the apoptosis induction genes GADD45A, CASP2, and CASP8 were up-regulated (**Paper II**) in relation to elevated arsenic concentrations, indicating arsenic-induced apoptosis of immune cells. A study conducted in Mexican children aged 6-14 years showed a positive association between U-As and the percentage of apoptotic cells in PBMC (Rocha-Amador et al. 2011). Higher rates of arsenic-induced mitochondrial-dependent apoptosis were also reported in PBMC in arsenic-exposed adults with skin lesions compared to unexposed individuals (Banerjee et al. 2008). Under normal conditions, apoptosis is important for immune cells to fight against pathogens. However, abnormal or induced apoptosis may result in immunodeficiency, autoimmunity, or malignant transformation (Thompson 1995).

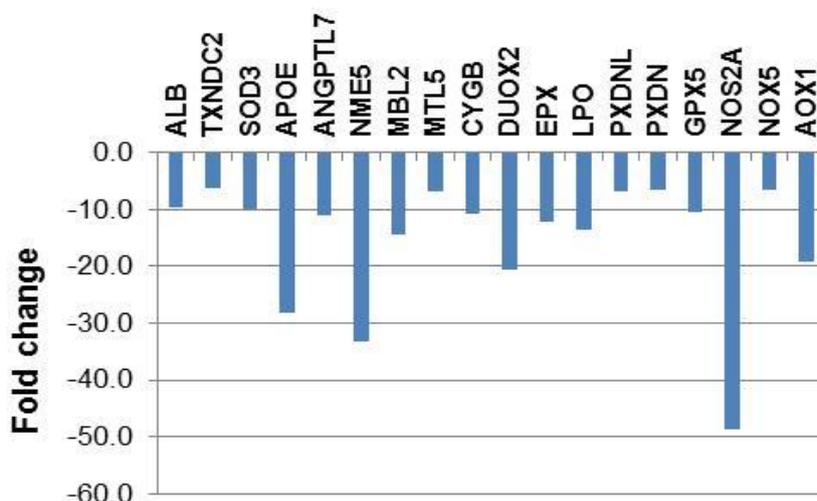


Figure 11: Genes involved in oxidative stress defense pathway in cord blood mononuclear cells in highly exposed newborns (cord blood arsenic, mean \pm SD, $19.3\pm 11.4 \mu\text{g/kg}$, and $n=6$) compared to those with low exposure ($1.1\pm 1.0 \mu\text{g/kg}$, $n=6$).

4.7.3 Arsenic exposure and cytokines in placenta, cord blood, and in plasma of children at 4.5 years of age

The placenta is the interface between fetal and maternal circulation and mediates the transport of gases, nutrients, and waste products. During a normal pregnancy, to protect the

maternal-fetal interface, the immune system shifts towards a Th2 cytokine response and, as a result, Th1 dependent cytokine production is suppressed. Regulatory T cells (Zenclussen et al. 2007) and NK cells (Saito et al. 2008) help to maintain a local environment where inappropriate allogeneic antigen stimulation is minimized to protect the fetus from being recognized as foreign (Calleja-Agius and Brincat 2008). Placental growth factor acts on dendritic cells and produces a down regulation of Th1 cytokines (Lin et al. 2007). However, we observed arsenic-induced elevated expression of Th1 pro-inflammatory cytokines (TNF- α , IFN- γ , and IL-1 β) both in placenta and cord blood (**Figure 12A and B**), indicating arsenic-induced disruption of the immune balance and increased inflammation at the maternal-fetal interface. Arsenic also accumulates in the placenta (Concha et al. 1998), and may give rise to toxic effects via oxidative stress (Massrieh et al. 2006), impaired transport of nutrients to the fetus, and also inhibit some antioxidant and hormonal activities (Lu et al. 2007). We also found that placental pro-inflammatory cytokines were highly correlated with the placental oxidative stress marker 8-oxoG (**Paper I**). The elevated oxidative stress in placenta may trigger the secretion of pro-inflammatory cytokines, which have been associated with preeclampsia and pre-term birth (Myatt 2006; Cindrova-Davies et al. 2007). Cord blood pro-inflammatory cytokines are used as a marker of early onset of infection in premature infants (Krueger et al. 2001).

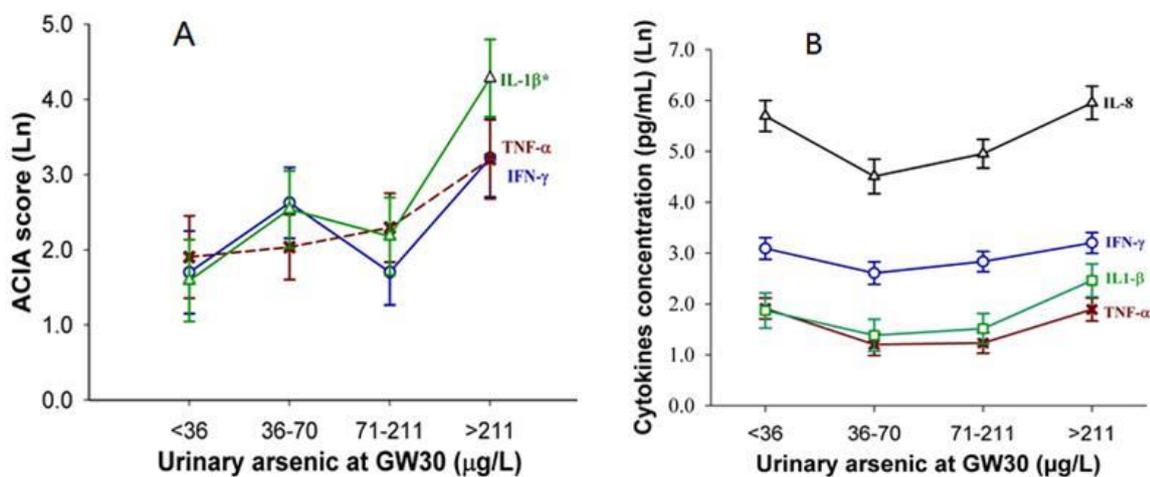


Figure 12: Maternal arsenic exposure and pro-inflammatory cytokines in (A) placenta and (B) cord blood.

The child is born with predominant Th2 (anti-inflammatory) immune responses. After birth, the immune system requires further maturation of Th1 (pro-inflammatory) cytokine responses to achieve effective host resistance to infections (Dietert and Zelikoff 2008). Therefore, maturation of immune cells, e.g. macrophages and CD4+ T cells, is required for children to appropriately develop cell-mediated immunity against pathogens and transformed cells (Dietert and Zelikoff 2008). In **Paper III**, we observed inverse associations between concurrent U-As and plasma cytokines IL-2 and TNF- α (in the highest tertile, regression coefficients (95% CI): -1.57 (-2.56, -0.57); and -4.53 (-8.62, -

0.42), respectively), both Th1 cytokines, but not with the other 25 cytokines, in children at 4.5 years of age. These results indicate that these children may be more susceptible to infections since suppression of Th1-dependent functions has been linked to infectious disease susceptibility (Obgaidze et al. 2005).

4.8 METHODOLOGICAL CONSIDERATIONS

4.8.1 Limitations of the study

In **Paper I** and **II**, we were able to include only the women who delivered in the hospital during day time, and those who had available placenta and cord blood (see section 3.2). Thus, the main limitation of these two studies was the small sample size (n=130). However, we did not observe any significant differences in basic characteristics with the exception of SES, when we compared the studied women, and the rest of the women enrolled in the MINIMat trial during the same study period (July 2002 to September 2003). Our studied women were slightly richer than the rest of the women enrolled in the MINIMat trial. In **Paper III**, we included 640 MINIMat children who were born between June 2003 to June 2004, with available urine and blood samples. Out of them, 577 children participated in the PPD skin testing. Here, we also compared the studied children with the rest of the children enrolled during the same study period in relation to their basic characteristics, and we did not observe any significant differences that might introduce selection bias.

We measured U-As using HPLC-HG-ICPMS with high accuracy and very low limit of detection (<1 µg/L). The CHRWs, who collected urine samples and other questionnaire based information, were not aware of the exposure status of the study participants. The study physicians, who recorded the induration size of the children (**Paper III**), were blinded regarding information about the exposure. We also collected morbidity information (2-4 weeks recall) (**Paper I-III**) based on maternal-reported signs and symptoms (acute respiratory infection and diarrhea). This is a common way to collect morbidity information in developing countries where most of the cases are not reported to or treated at the health care facilities (Feikin et al. 2010). Icdrr,b has been conducting respiratory infections surveillance in the study area since 1988 (Fauveau et al. 1992). Therefore, we believe that mothers are quite familiar with the key symptoms of respiratory infections and diarrhea, both of which are prevalent in children.

Due to the limitations of the samples available we were not able to follow the same studied women (n=130) in our second study (**Paper II**). Another drawback was that all newborns were not followed up in their childhood at 4.5 years of age, although the sample size was much bigger (n=640). We did not measure CD4+ cells or CD4+ T regulatory cells in the placenta which could have allowed us to evaluate the immune status in placenta in a much better way. Another limitation was that we were not able to do sterile cell culture experiments, e.g., lymphocyte proliferation response, antigen stimulation assays to measure cytokines, due to lack of appropriate facilities (such as a biosafety cabinet or tissue culture hood and CO₂ incubator) in the study area.

4.8.2 Confounding

One of the main strengths of the studies (**Paper I-IV**) was the availability of data on relevant covariates (e.g. SES, morbidity, season of birth), which could be used to adjust for possible confounding. In **Paper I-IV**, potential confounders were selected if they were associated with both exposure and outcome, and if they changed the estimate more than 5%, or if they were considered biologically relevant risk or protective factors. The SES came out as an important confounding factor in the analysis of all four studies included in this thesis. In children at 4.5 and 9 years of age we also measured other elements in urine, including cadmium and lead. However, we did not observe any clear confounding effects by these metals. The knowledge about the co-existence of other contaminants in this rural setting is relatively limited so far.

4.8.3 Arsenic metabolism efficiency

The fractions of inorganic arsenic and MMA in urine are considered as susceptibility factors for several arsenic-related health effects as this has been previously shown in adults (Vahter and Concha 2001; Vahter 2009). However, we did not observe any correlation between methylation efficiency and immune-related health outcomes in children. This is probably due to the fact that the children methylation efficiency is influenced by several other factors than in adults (our preliminary observation). Further studies are required to explore differences in arsenic metabolism efficiency and their implications in adults and children.

5 GENERAL DISCUSSION

The studies in this thesis were conducted in Matlab, a rural part of Bangladesh and included pregnant women and their children with generally low socioeconomic conditions, high prevalence of malnutrition, and wide use of arsenic contaminated groundwater for drinking and cooking purposes. The immune system develops over an extended period of time during in early life. The period from pregnancy to early childhood is considered as the critical windows of immune vulnerability to toxicants (Dietert and Zelikoff 2008). Exposure to immune-toxicants such as arsenic during this period exerts detrimental effects on the developing immune system, as shown by the studies presented in this thesis. The extent of exposure of pregnant women and their children in Bangladesh, as well as in other countries (Vahter et al. 2006; Vahter 2009; Majumdar and Guha Mazumder 2012; Farzan et al. 2013), highlights the public health significance of our findings.

We investigated the effects of prenatal and childhood arsenic exposure on the developing immune system. The results indicated that arsenic affects immune cells and their functions, in addition to increasing inflammation (**Figure 13**). In **Paper I** and **II**, we observed that prenatal arsenic exposure reduced T cells in placenta and naïve T cells in cord blood. The mechanism could be arsenic-induced oxidative stress, inadequate defense against oxidative stress, induction of apoptosis, or a combination of these. These results indicated that the children were born with a low number of T cells which could make them more susceptible to infections. In **Paper III**, we observed that early childhood arsenic exposure was related to impaired cell-mediated immune function in children at 4.5 years of age, probably due to arsenic-induced reduction of T cells and macrophages, and/or through impairment of their functions. This implies that the children cannot cope with the low number of T cells due to continuous exposure. It can also be speculated that concurrent exposure may inhibit the number of peripheral T cells or their function. In **Paper IV**, we found evidence that prenatal arsenic exposure had stimulatory effects on B cells (high production of IgG antibodies) in children at 9 years of age. This result implies that arsenic changed the programming of B cells during fetal life, probably via alteration of the epigenome. Additionally, we observed an indication of impaired vaccination response in children at 9 years of age in relation to early childhood arsenic exposure, probably due to arsenic-induced impaired development of antigen-specific T cells.

Our results support the concept of early life arsenic exposure affecting health later in life (Vahter 2009; Farzan et al. 2013). A study conducted in a Chilean population (aged 30-49 years) showed that those born before and during the high arsenic exposure period (1958-1970), with probable exposure in early childhood and *in utero*, had a higher risk of bronchiectasis than the rest of the Chilean population (Smith et al. 2006). The standardized mortality ratio for bronchiectasis was eight times higher in adults who were exposed *in utero* and early childhood compared to those who were exposed only in early childhood. Bronchiectasis is caused through ineffective defense against airway inflammation, probably due to immunosuppression (Whitters and Stockley 2012). Recent studies in children showed

that *in utero* and early childhood arsenic exposures were associated with decreased lung function and increased chronic respiratory symptoms (Smith et al. 2013; Recio-Vega et al. 2014). Thus, it is probable that early life exposure changes the immune programming or alters the immune maturation, which leads to later life effects (**Figure 13**). We observed some of the arsenic related immune effects even below 50 µg/L of maternal urinary arsenic. In particular, these levels of arsenic reduced the number of T cells and induced oxidative stress in placenta. These results further highlight the importance of changing the local drinking water standard in Bangladesh and adopting the WHO guideline value of 10 µg/L immediately.

The studied children were exposed to arsenic prenatally and many of them continued being exposed during their postnatal life. Future follow-up will help to better understand the later life health effects in relation to early life and long term exposure. Also, further studies are required to confirm the adverse effects of early life arsenic exposure on the developing immune system in other settings. The current evidence together with the other documented effects of arsenic, stress the need to give high priority to reducing the exposure, particularly for pregnant women and their children. If more effective mitigation strategies are delayed further, we may observe even higher incidence of morbidity and mortality due to infectious disease, and possibly also other chronic diseases such as cancer, cardiovascular diseases, and diabetes in the arsenic affected area.

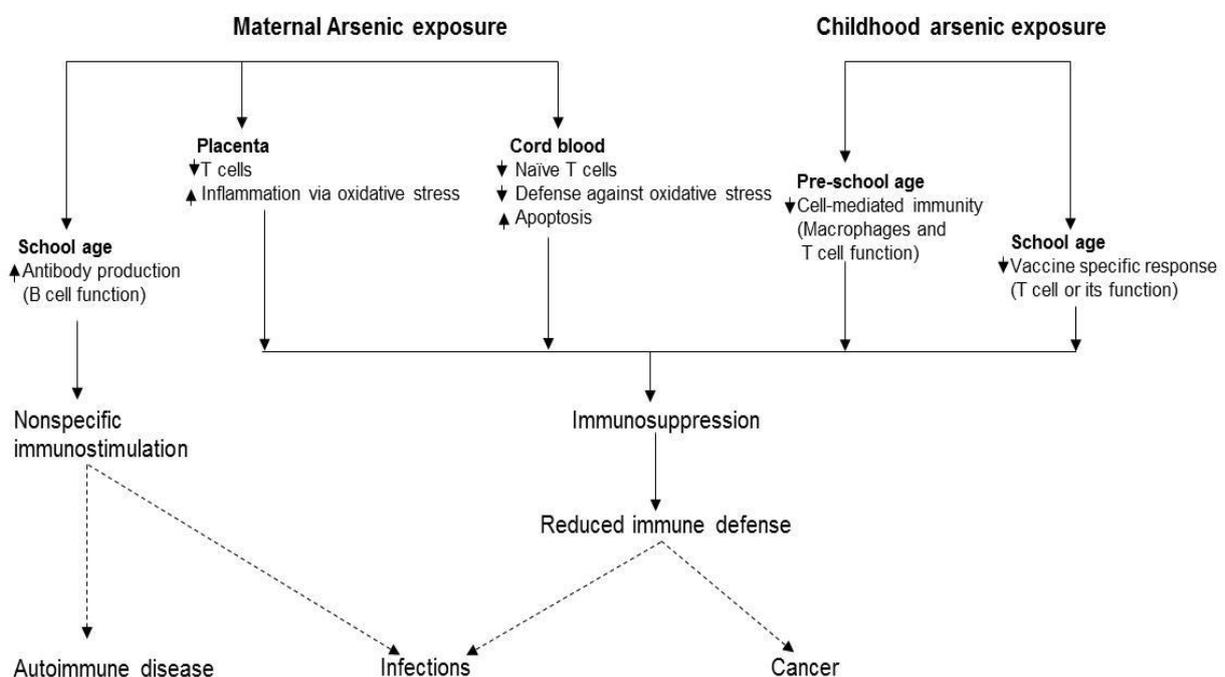


Figure 13: Schematic flow chart of arsenic-induced alterations of immune responses. Upward arrows indicate increases and downward arrows indicate decreases. Dashed lines indicate how such immune effects could contribute to health effects.

6 CONCLUSIONS

Despite the arsenic mitigation activities that have been conducted during the last decade in the study area, the children are still exposed to elevated levels of arsenic, mainly via drinking water and food. We found evidence that this elevated arsenic exposure seemed to adversely affect the developing immune system. Maternal arsenic exposure during pregnancy reduced naïve T cells in cord blood, indicating that the children were born with a low number of T cells, which could in turn make them more susceptible to infections. Early childhood arsenic exposure appeared to reduce cell-mediated immune function in children at 4.5 years of age, indicating that the children cannot cope with the number or function of T cells due to continuous childhood exposure. We also found preliminary evidence of arsenic-induced reduction of mumps-specific vaccination response, probably due to low number of T cells or impaired T cell function. Maternal arsenic exposure during pregnancy seemed to increase total plasma IgG in children at 9 years, indicating that arsenic altered fetal B cell programming. All of these effects of early life arsenic exposure on T cells, and T cell and B cell-associated immune function imply biological plausibility to various types of immune-related diseases in childhood and perhaps also later in life.

7 MAIN FINDINGS

- ✚ Pregnant women and their children in Matlab, Bangladesh, are exposed to elevated arsenic exposure, mainly via drinking water and food.
- ✚ The exposure has decreased slightly over time due to arsenic mitigation activities but is still highly elevated.
- ✚ Maternal arsenic exposure during pregnancy was associated with reduced placental T cells and increased inflammation in placenta and cord blood, possibly related to arsenic-induced oxidative stress.
- ✚ Maternal arsenic exposure during pregnancy was associated with reduced thymic function (naïve T cells) in newborns, possibly via impaired cellular anti-oxidative defense systems or increased apoptosis in thymocytes or naïve T cells.
- ✚ Childhood arsenic exposure was associated with reduced cell-mediated immunity in children at 4.5 years of age, possibly via impaired T cell and /or macrophage function.
- ✚ Prenatal arsenic exposure showed a stimulatory effect on B cells (high production of antibodies) in children at 9 years of age, possibly due to arsenic-induced alteration of B cell programming.
- ✚ Early childhood arsenic exposure appeared to reduce mumps-specific vaccination response in children at 9 years of age, possibly due to arsenic-induced impaired development of antigen-specific T cells.

8 FUTURE RESEARCH

- ✚ Continue follow-up children within the study prospectively to explore potential health effects of early life and long term arsenic exposure later in life.
- ✚ Further studies are required to investigate the effects of arsenic on T regulatory cells as they are involved in maintaining immune tolerance.
- ✚ Further studies are necessary to evaluate epigenetic changes of immune related genes in relation to maternal arsenic exposure.
- ✚ As arsenic exposure impaired the immune response to infections, studies evaluating effects of arsenic on anti-microbial peptides and complement proteins are needed.
- ✚ Further studies are required to evaluate immune-related health effects in relation to low levels of arsenic exposure in drinking water (<50 µg/L).
- ✚ Studies that evaluate the frequency of all immune cells in relation to arsenic exposure are also required.
- ✚ Arsenic-induced stimulatory effects on B cells should be further validated.
- ✚ Vaccinations against a pathogen for which the study population is completely naïve should be used to evaluate arsenic-related effects on vaccination response.
- ✚ Further studies are required to evaluate immune-related health effects of arsenic in relation to arsenic metabolism efficiency at different life stages.
- ✚ Despite extensive mitigation activities targeting drinking water sources and subsequently reduced exposure from these sources, U-As levels in the population remain highly elevated, indicating arsenic exposure from food as well. It is important to evaluate the influence of various foods to the total arsenic exposure.

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