Effect of exposure to arsenic on immune function and anemia status in children in a birth cohort study

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Tania Mannan

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

FACULTY OF BIOLOGICAL SCIENCES

UNIVERSITY OF DHAKA

DHAKA, BANGLADESH

MAY 2019

Effect of exposure to arsenic on immune function and anemia status in children in a birth cohort study

BY

Tania Mannan

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

Faculty of Biological Sciences,
Department of Biochemistry and Molecular Biology,
University of Dhaka,
Dhaka, Bangladesh.

Registration Number:
28/2014-15

Certificate

This is to certify that Tania Mannan has conducted her thesis work entitled, "Effect of exposure to arsenic on immune function and anemia status in children in a birth cohort study" under my supervision for the fulfillment of the degree of 'Doctor of Philosophy in Biochemistry and Molecular Biology' from the University of Dhaka. The research work has been conducted in the Immunobiology, Nutrition and Toxicology Laboratory, Infectious Diseases Division, icddr,b and supervised by Dr. Rubhana Raqib. The work or any part of the thesis has not been submitted anywhere for any other degree.

Supervisors

Ishtiaq Mahmud, PhD
Professor
Department of Biochemistry & Molecular Biology
University of Dhaka
Cell: +8801911022531
ishtiaq51@yahoo.com

Zakir Hossain Howlader, PhD
Professor
Department of Biochemistry & Molecular Biology
University of Dhaka
Cell: +8801716409601
hhzakir@yahoo.com

Rubhana Raqib, PhD
Senior Scientist and Head,
Immunobiology, Nutrition and Toxicology Laboratory
Infectious Diseases Division
icddr,b
Cell: +8801713040942
rubhana@icddrb.org

Supervisors:

Professor Ishtiaq Mahmud
Department of Biochemistry and Molecular Biology
University of Dhaka

Professor Zakir Hossain Howlader
Department of Biochemistry and Molecular Biology
University of Dhaka

Dr. Rubhana Raqib
Senior Scientist and Head,
Immunobiology, Nutrition and Toxicology Laboratory
Infectious Diseases Division
icddr,b

Women at a village pond in Matlab, Bangladesh, washing utensils and vegetables. The woman on the right is putting a sari filter onto a water-collecting pot (or kalash) to filter water for drinking. (Picture courtesy of Anwar Huq, University of Maryland Biotechnology Institute, Baltimore, Maryland, United States.)

https://www.researchgate.net/publication/9003496/figure/fig1/AS:339983920582656@1458069891273/Women-at-a-Village-Pond-in-Matlab-Bangladesh-Washing-Utensils-and-Vegetables-The-woman.png

To my Family

Acknowledgements

The long road that brought me to this Doctoral Thesis has been paved with the support of a number of people, to whom I owe my deepest gratitude. First and foremost, it was an honor to have worked with icddr,b's MINIMat project; I consider myself fortunate, having received warmth and assistance from everyone I interacted with. Finally, I would like to thank the MINIMat research team and icddr,b overall for the great work they are executing.

Likewise, I would like to express my sincere thanks to and appreciation for my supervisor, Prof. Dr. Ishtiaq Mahmud, who has always given me academic advice, mental support and taken the time and effort to edit my thesis thoroughly.

My other supervisor Dr. Rubhana Raqib who made all of this possible, has taught me so much about academic research. I am really grateful to her for giving me the opportunity to complete my thesis in her lab and for introducing me to the world of professional research. I have enormous respect for her, both as a scientist and as an individual. Her endless encouragement and helpful insights have granted me the confidence to continue my studies and work. Under her guidance I learnt how to approach and pursue interesting leads, even when the implications of the results seemed uncertain. The most unique thing about her is that, every single day I spent with her, I learnt something new. I am indebted to her in so many ways that thanking her does not seem to be enough of an appreciation.

Furthermore, it is indeed, with great pleasure, that I express my heartfelt gratitude to Prof. Dr. Zakir Hossain Howlader for his time, invaluable academic instruction, and for his understanding during difficult times.

I am grateful to Prof. Dr. Laila N Islam for her expert guidance which pushed me to rethink my research in a more intense manner.

With all my heart, I would like to express my thankfulness to Prof. Zeba Islam Seraj, Chairperson, Department of Biochemistry and Molecular Biology, University of Dhaka for giving me the opportunity, necessary infrastructural facilities and moral support to satisfy my desire to gain higher education. I also acknowledge the cooperation of the teachers, research scholars, students and non–teaching staffs of the Department of Biochemistry and Molecular Biology at the University of Dhaka.

Evana Akhtar deserves special thanks and appreciation for her excellent support with the laboratory analysis. I would like to extend my best and sincere wishes for her PhD research. I am also thankful to the other members of the Immunobiology, Nutrition and Toxicology Laboratory of Infectious Diseases Division of icddr,b. This work would never have been possible without their help, patience and expertise. Not to mention, Mr. Ahsanul Haq (Shohag), deserves special recognition for his support during the process of database and statistical analysis.

I would also like to acknowledge all the co-authors for their contribution to the included studies, especially, Dr. Sultan Ahmed for his valuable input. My sincere thanks also goes out to the women and children of *Matlab Bangladesh,* who participated in my trials.

My special thanks to and deep sense of gratitude for Prof. Dr. Faridul Alam, Vice-Chancellor, Bangladesh University of Health Sciences (BUHS) for giving me the permission and scope to do research and teach simultaneously. I acknowledge and am truly grateful for the mental support rendered by Prof. Dr. Rosy Sultana, Head of the Immunology Department, BUHS and my fellow colleagues.

I am deeply thankful to all my friends who have always given me the moral support to accomplish and succeed in my work.

I dare not use mere words to reduce the extent of gratitude I have for my parents, who have always believed in me, raised me with their spirit and encouraged me to reach this level with never-ending love. They gave me their all, and taught me the significance of education, focus, and dedication. My father would be the happiest person in the world to see me receive my PhD. I miss you everyday Abba!

I would not be half the person I am today, without my mother's understanding and unconditional love, care and endless sacrifice. She has always believed in me and in all that I do, both in academia and in life. I will try my best to fulfill your expectations till the last day of my life, Amma!

Life blessed me with two children, Modhurima and Ahan, who have shown wisdom and maturity beyond their years, throughout this challenging process. Not to mention, my Shonama, Modhubonti, has always been supportive in taking the effort to care for her siblings. All my love to our children; with my whole heart, I wish you a bright and peaceful life! Throughout my life, my two elder sisters have been a constant source of moral support. Especially my eldest sister, Dr. Fouzia Mannan, who has taught me positivity and perseverance even during the most challenging of circumstances. I would also like to acknowledge my in-laws, who have always believed in me and given me moral support and affection.

Last but not the least, I reckon it would have been all but impossible to pursue my research career, without the immense love, support and encouragement from my husband and friend, Shoaib Chowdhury. I would like to take this opportunity to thank him for his patience and his unremitting belief in my abilities.

Thank you all, for the endless love and support towards making my dream come true!

Warmly,

Tania Mannan

ABSTRACT

Environmental exposure to arsenic is a global health concern which is linked to a number of diseases causing adverse health effects. Chronic arsenic exposure affects an estimated number of 200 million people worldwide through contaminated groundwater and food. In Bangladesh, the arsenic problem is very devastating and related health problems have yet not been fully investigated. There is consistent evidence that chronic arsenic toxicity may cause cancer and numerous other pathological effects in humans as well as immune toxicity. Several cross-sectional studies have indicated that arsenic adversely affects the immune system in both children and adults. However, very few longitudinal studies in human have further indicated that the adverse effects of the toxicant start from *in utero*. Exposure to arsenic has also been found to increase risk of anemia by disrupting cell membranes and declining erythrocyte survival. However, findings from epidemiological studies are equivocal in support to this association.

This thesis delineates the plausible effects of chronic arsenic exposure from *in utero* to pre-adolescent age and the detrimental effects on fetal and infant health and development, as well as undesirable health effects later in life. The overall objective of the present study has been focused to investigate the impact of prenatal and concurrent exposure to arsenic in 9 years old school-going children on anemia and immune function. The studies were nested into a large food and micronutrient supplementation trial conducted in a rural area of Bangladesh called Matlab. Mother-child pairs were followed from early gestation to 9 years. Elevated arsenic concentrations in groundwater are common in Matlab where 95% of the population uses hand-pumped tubewell water as their main source of drinking water with 43% of the wells exceeding the World Health

Organization (WHO) guideline value of 10 µg/L, and 34% exceeded the Bangladesh standard of 50 µg/L.

Arsenic exposure in the present studies was assessed based on the concentrations of the sum of inorganic arsenic and its methylated metabolites (monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)) in maternal urine during pregnancy and child urine at 4.5 and 9 years of age (Urinary arsenic: U-As). Hemoglobin (Hb) was measured in whole blood in pregnant women at gestation week (GW) 14 and 30, and in children at 4.5 and 9 years of age. Other markers that were studied in 4.5 and 9 years old children were- anemia related biomarkers (soluble Transferrin Receptor (sTfR), ferritin, vitamin B_{12}, folate, vitamin A, hepcidin, zinc), inflammatory markers (C-reactive protein (CRP)), oxidative stress marker (8-hydroxy-2'-deoxyguanosine (8-OHdG)) in plasma, and immune marker (signal joint T cell receptor excision circles (sjTRECs) and indicator of senescence (telomere length (TL)) in peripheral blood mononuclear cells (PBMC).

No effects of arsenic exposure on Hb levels and anemia biomarkers was found in children. About 28% of the women were found to be anemic at GW14, 35% at GW30 and 23% at both time points. The prevalence of anemia was low in 4.5 years old children (5%) that somewhat increased in 9 years old (15%). Only 2.5% children had iron deficiency anemia according to sTfR cut-off at 9 years. The risk (Odds Ratio (OR)) of being anemic in 9 years of age was 1.8 and 2.3 fold higher if their mothers were anemic at either GW14 or GW30, respectively, compared to non-anemic mothers. The chances of having anemia at 9 years was 3 times higher if the mothers were anemic at both time points (OR=3.05). This risk increased about 6 times (OR=5.9) when the children were additionally anemic at 4.5 years. No impact of maternal anemia was observed at 4.5 years of age. Multiple Micronutrient (MM) supplementation significantly improved body mass index-for-age z-score (BAZ) and reduced markers of inflammation in 9 years old children.

In multivariable-adjusted spline regression analyses, both prenatal (U-As at GW8) and childhood arsenic exposure (U-As at 4.5 and 9 years of age) above U-As of 45 µg/L (spline knot) strongly reduced TL and sjTRECs at 9 years. However, concurrent U-As below the spline knot was significantly positively associated with TL and sjTRECs at 9 years of age. It is plausible that there is a critical threshold of arsenic exposure beyond which exposed cells either undergo telomere attrition or elongation of TL. In 9 years old children, fraction of MMA above spline knot 7% were significantly inversely associated with both TL and sjTRECs reflecting increased toxicity due to less-efficient arsenic metabolism. Prenatal and childhood arsenic exposure were positively associated with 8-OHdG at 9 years which in turn was inversely associated with sjTRECs at 9 years. However, adjustment with 8-OHdG did not change the estimated association of U-As with sjTRECs suggesting that the mechanisms of 8-OHdG-mediated oxidative damage of naïve T cells may be distinct from arsenic-induced oxidative damage with little overlap.

We can, therefore, summarize that chronic arsenic exposure did not appear to have any impact on anemia status of children. Anemia during pregnancy and early childhood are important risk factors for the occurrence of anemia in school-age children. Maternal supplementation during pregnancy with MM had beneficial effects on child nutritional status and reduced markers of inflammation in the children. Chronic arsenic exposure from early life can result in TL attrition and lower production of naïve T cells potentially leading to immunosenescence and immunodeficiency. The adverse effects of arsenic on sjTRECs and TL may be mediated by pathways other than 8-OHdG-induced oxidative stress, e.g. impairment in DNA repair enzymes and synthesis.

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-II:

I. **Mannan T**, Ahmed S, Akhtar E, Roy AK, Haq MA, Roy A, et al. (2016) Maternal micronutrient supplementation and long term health impact in children in rural Bangladesh. PLoS ONE 11(8): e0161294. doi:10.1371/journal.pone.0161294

II. **Mannan T**, Ahmed S, Akhtar E, Ahsan K B, Haq A, Kippler M, Vahter M, Raqib R. Associations of arsenic exposure with telomere length and naïve T cells in childhood–A birth cohort study. Toxicol Sci. 2018;164(2):539-549. doi: 10.1093/toxsci/kfy105.

LIST OF TABLES

Table no.	Title	Page no.
Table 1	Baseline characteristics of the study participants	35
Table 2	Regression analysis of urinary arsenic concentration at gestation week 8, 4.5 and 9 years with hemoglobin at gestation week 14 & 30, 4.5 and 9 years of age	37
Table 3	Plasma biomarkers in school-age children in rural Matlab	38
Table 4	Indicators used in this thesis to define anemia and micronutrient deficiencies in school-age children	39
Table 5	Analysis of covariance of child nutritional status and plasma biomarkers in different supplementation groups	40
Table 6	Linear regression analyses between hemoglobin concentrations and plasma biomarkers in all children and boys and girls separately	42
Table 7	Regression analysis of urinary arsenic concentration at gestation week 8, 4.5 and 9 years and arsenic metabolites at 9 years with telomere length at 4.5 and 9 years of age	47
Table 8	Regression analysis of urinary arsenic concentration at gestation week 8, 4.5 and 9 years and arsenic metabolites at 9 years with sjTRECs at 4.5 and 9 years of age	51
Table 9	Regression analysis of urinary arsenic concentration at gestation week 8, 4.5 and 9 years with 8-OHdG at 4.5 and 9 years of age	52

LIST OF FIGURES

Figure no.	Figure legend	Page no.
Figure 1	TRECs are circular DNA fragments. They are generated when a section of chromosome 14 (14q11-2), which contains genes responsible for coding T-cell receptors, rearranges. The TRECs are amplified by PCR	9
Figure 2	Map of Bangladesh and Matlab study area	19
Figure 3	Flow diagram depicting the recruitment of children in the study nested in the MINIMat birth cohort study in rural Bangladesh carried out in 2012–2013	22
Figure 4	Associations between urinary arsenic concentration (U-As) (log_2-transformed) at GW8, 4.5 and 9 years and telomere length (A, B and C) and signal-joint T cell receptor excision circles (sjTRECs) (log_2-transformed) (D, E and F) in 9 years old children. In the scatter plots, the solid line represents a Lowess (locally weighted scatter plot smoothing) moving-average curve for the raw data	32
Figure 5	Association of telomere length between 4.5 and 9 years of age (n=211). In the scatter plots, the solid line represents a Lowess (locally weighted scatter plot smoothing) moving-average curve for the raw data	36
Figure 6	The odds of having anemia at 9 years of age if the mother were anemic at GW14 or GW30 or if the children were anemic at 4.5 years of age	45
Figure 7	Linear mixed effects models of U-As over time (4.5 and 9 years of age) on outcome changes of telomere length (A) and sjTRECs (B) in children with a spline knot introduced at 5.5 (corresponding to 45 µg/L of U-As). The models were adjusted with covariates (child age, height-for-age z-score, gender, family SES at 4.5 and 9 years of age, plasma CRP concentration at 9 years, mother's education and U-As at GW 8	49

LIST OF ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
As	Arsenic
As3MT	Arsenic methyltransferase
As(III)	Arsenite
As(V)	Arsenate
ATSDR	Agency for Toxic Substances and Disease Registry
BAZ	Body mass index-for-age z-score
BMI	Body mass index
CBC	Complete blood count
CDC	Centers for disease control and prevention
CHRW	Community health research worker
CI	Confidence interval
cm	Centimeter
CRP	C-reactive protein
CV	Coefficient of variation
DMA(III)	Dimethylarsinous acid
DMA(V)	Dimethylarsinic acid
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay

ESR	Erythrocyte Sedimentation Rate
Fe	Iron
Fe30F	30 mg iron and 400 µg folic acid supplement
Fe60F	60 mg iron and 400 µg folic acid supplement
Fl	Femtoliter
g/l	Grams per liter
GSH	Glutathione
GW	Gestational week
HAZ	Height-for-age z-score
Hb	Hemoglobin
HDSS	Health and demographic surveillance system
HG	Hydride generation
HPLC	High-performance liquid chromatography
HSC	Hematopoietic stem cell
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
ID	Iron deficiency
IDA	Iron deficiency anemia
IgA	Immunoglobulin A

IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
kb/dg	Kilo base pair/diploid genome
Kg	Kilogram
Km	Kilometer
LSD	Least significant difference
MALT	Mucosal-associated lymphoid tissue
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
µg	Microgram
µg/L	Micrograms per liter
µL	Microliter
µmol/l	Micromole per liter
mg	Milligram
mL	Milliliter
MINIMat	Maternal and infant nutrition interventions in Matlab
MM	Multiple micronutrients supplement including 15 micronutrients
MMA(III)	Monomethylarsonous acid
MMA(V)	Monomethylarsonic acid

nmol/l	Nanomole per liter
ng	Nanogram
ng/mL	Nanograms per milliliter
NCD	Non-communicable diseases
NRC	National Research Council
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
pmol/l	Picomole per liter
RBC	Red blood cell
RDW	Red cell distribution width
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SAM	S-adenosylmethionine
SD	Standard deviation
SES	Socioeconomic status
SG	Specific gravity
sTfR	Soluble transferrin receptor
sjTREC	signal joint T cell receptor excision circle
TCR	T cell receptor
TL	Telomere length
Th1	T helper type 1
tIgE	Total plasma IgE

tIgG	Total plasma IgG
U-As	Urinary arsenic, sum of iAs, MMA and DMA
UNICEF	United Nations Children's Fund
WAZ	Weight-for-age z-score
WHO	World Health Organization
Zn	Zinc

TABLE OF CONTENTS

Acknowledgements		**i-ii**
Abstract		**iii-v**
List of Scientific Papers		**vi**
List of Tables		**vii**
List of Figures		**viii**
Abbreviations		**ix-xiii**
Chapter 1: Introduction		**1**
1.1	**Arsenic in the Environment and its Effects on Human Health**	**2**
1.1.1	**Abundance, forms, and distribution**	**2**
1.1.2	**Use and source**	**3**
1.1.3	**Human exposure to arsenic**	**3**
1.1.4	**Arsenic exposure in Bangladesh**	**4**
1.1.5	**Arsenic biotransformation**	**5**
1.1.6	**Toxicity and health effects**	**6**
1.1.6.1	**Mechanisms of arsenic toxicity**	**7**
1.1.7	**Immune system and its function**	**7**
1.1.8	**Signal-joint T-cell receptor excision circle (sjTREC)**	**8**
1.1.8.1	**Immunosenescence**	**9**
1.1.9	**Immunotoxicity**	**10**
1.1.10	**Impact of arsenic on immune function**	**10**
1.1.11	**Immunosenescence /Telomere length and arsenic**	**11**
1.2	**Anemia and Micronutrient Deficiency in Children**	**12**
1.2.1	**Impact of arsenic on anemia and micronutrients**	**13**
1.3	**Aims of the thesis**	**15**

Chapter 2: Materials and methods 17
2.1 Study setting 18
2.2 Study design and population 19
2.3 Data collection and sampling 23
2.4 Laboratory analyses 25
2.4.1 Assessment of plasma biomarkers 25
2.4.1.1 Hemoglobin and anemia 26
2.4.2 Urinary arsenic 26
2.4.3 Estimation of sjTRECs 27
2.4.4 Estimation of Telomere length 28
2.4.5 Oxidative stress marker 29
2.5 Ethical consideration 29
2.6 Statistical analyses 29
Chapter 3: Results 33
3.1 General characteristics of women and children 34
3.2 Hb and arsenic exposure 36
3.3 Status of nutritional biomarkers (Paper I) 37
3.4 Influence of maternal supplementation on child growth and inflammatory markers (Paper I) 39
3.5 Association of Hb with plasma biomarkers and effect of maternal micronutrient supplementation (Paper I) 41
3.6 Long term impact of maternal anemia in school-age children (Paper I) 43
3.7 Chronic arsenic exposure and telomere length (Paper II) 46
3.8 Chronic arsenic exposure, sjTRECs and immune cells (Paper II) 50
3.9 Arsenic exposure, oxidative stress, telomere length and sjTRECs (Paper II) 52
Chapter 4: Discussion 53
5 Conclusions 66
6 References 68

CHAPTER 1
INTRODUCTION

There is a growing interest on the potential effects of arsenic exposure on maternal and child health focusing on pregnancy outcomes, child health including immune function and development. The present thesis emphasized on the effects of prenatal and concurrent exposure to arsenic on immune function and anemia in 9 years old children.

Mounting evidence suggests that chronic arsenic exposure during pregnancy and early life is associated with increased risk of infectious diseases, in particular respiratory and gastrointestinal infections (1-4). Several cross-sectional studies have indicated that arsenic adversely affects the immune system in both children and adults (5). However, very few longitudinal studies in human have further indicated that the adverse effects of the toxicant start from *in utero* (3, 6-10).

Anemia and micronutrients deficiencies in mother and children are common in low-income settings. These are partly due to maternal malnutrition that may impair child health and development. Few epidemiologic studies have investigated the impact of arsenic exposure on anemia despite its high prevalence in the regions which experience groundwater arsenic contamination. Also, data is scarce on the consequence of long-term effects of maternal nutrition supplementation on nutritional status in school going children.

1.1 Arsenic in the Environment and its Effects on Human Health

1.1.1 Abundance, forms, and distribution

Arsenic has a long and evil history and is among the ten most important chemicals of major public health concern according to World Health Organization (WHO). It occurs naturally in the environment in a wide variety of forms such as elemental, inorganic, organic and gas. It is mostly present in the trivalent or pentavalent forms when it combines usually with sulfur and other metals. The main oxidation states are arsenite (AsIII) and arsenate (AsV) in reducing and oxygenated

conditions respectively (11, 12). The most common inorganic forms in water are arsenite and arsenate, whereas arsenobetaine, arsenocholine, and arsenosugars are the important organic forms in certain foods, particularly in sea foods (11). Through natural or anthropogenic processes arsenic can enter the environment such as mining industry, agriculture, wood preservation activities, coal combustion etc. (13). The emission of arsenic into the atmosphere is due to these actions and it is redistributed on the earth's surface by means of dry fallout and rain. Arsenic is also mobilized by dissolution in water (14).

1.1.2 Use and source

In present and ancient context, it is known to be used in animal feed (as an additive to promote growth and prevent infections of animals), ceramics, glass, computers, semiconductors, herbicides, insecticides, pesticides, rodenticides, metallurgy, wood preservatives, dyes, and for many other applications (15, 16). Metallic arsenic is used in the manufacture of alloys generally with lead (e.g. in lead acid batteries) and copper. Gallium arsenide is used in high-speed semiconductor devices because of its light-emitting and electromagnetic properties (17). Arsine gas is used in computer chips and fiber optics as a doping agent (18). There were some medicinal applications of arsenic till the 1970s, for instance, the use of inorganic arsenic in the treatment of leukemia, psoriasis and chronic bronchial asthma, and organic arsenic in antibiotics for the treatment of spirochetal and protozoal disease (19). Arsenic originating from different applications contribute to anthropogenic arsenic pollution of the environment as well as to the naturally present arsenic (naturogenic pollution).

1.1.3 Human exposure to arsenic

The most important source of human arsenic exposure is drinking groundwater (20, 21). The WHO guideline of arsenic in drinking water is 10 μg/L. Presently arsenic above this value is

recognized as a public health problem in many countries, especially in the Southeast Asia, including India, Bangladesh and China (22). Bangladesh and India have retained the earlier WHO guideline of 50 μg/L as their standard of arsenic in drinking water (23, 24). An estimated number of 200 million people worldwide are chronically exposed to arsenic through contaminated groundwater above 10 μg/L (21). The accumulations of arsenic in surface soils are due to the use of arsenic-contaminated ground water for irrigation which can cause bio-accumulations of arsenic in edible plants and crops (25).

Even at low exposure levels, inorganic arsenic (iAs), the main form in both drinking water and rice, is a potent carcinogen and multi-organ toxicant for human (18, 26). Seafood is another source of arsenic exposure which mainly contains organic forms and this form is considered to be less toxic than inorganic. People may be exposed via inhalation of arsenic-containing particulates in areas with industrial emissions (27). Individuals can also be exposed to arsenic by contaminated water used in food preparation, smoking tobacco, eating poultry that were fed with organic arsenicals, and industrial processing (19, 28, 29).

1.1.4 Arsenic exposure in Bangladesh

In Bangladesh, people almost entirely depend on groundwater sources for drinking water and cooking. The increased use of groundwater for consumption made an outstanding achievement in reducing the scale of cholera and diarrheal diseases and infant mortality in the 1970s and 80s (30). However, access to safe drinking water was radically reduced due to the incidence of natural arsenic in groundwater and its exposure throughout the country especially in the southern half. In Bangladesh, around 22 million people (31) are affected by arsenic-contaminated water above the national standard. WHO described the arsenic crisis in Bangladesh as “the largest mass poisoning of a population in history.” Recent studies have shown that due to use of water from shallow pumps

for irrigation purposes, arsenic has entered the food chain affecting an even larger population (32, 33). Massive efforts have been carried out to reduce the burden (34-36). In 2009, country-wide water arsenic screening revealed a substantial improvement showing only 13.4% of the investigated 14,442 water sources exceeding the Bangladeshi drinking water standard of 50 μg/L of arsenic, however, the concentrations varied considerably across different areas (31).

1.1.5 Arsenic biotransformation

The major site of arsenic uptake and biotransformation is the human liver (37). After absorption of ingested iAs in the gastrointestinal tract both in humans and animals (38, 39), arsenate is first reduced in the blood (40), and then methylated in the liver. Arsenate is reduced to arsenite in a reaction considered mostly to be dependent on glutathione (GSH) or other endogenous reductants. Arsenite then undergoes an oxidative methylation to form monomethylarsonic acid (MMA^V). MMA^V is reduced to MMA^{III} before a subsequent oxidative methylation step yielding dimethylarsinic acid (DMA^V). *S*-adenosylmethionine (SAM) acts as the methyl donor and Cyt19 (As3MT, arsenic methyltransferase) as the catalyst (41). Little is known about the in vivo reduction of DMA^V to DMA^{III} (42). The average proportions of the metabolites in human urine are 10-30% iAs, 10-20% MMA and 60-80% DMA (43). Exposure to iAs is measured by the sum of these metabolites, and methylation capacity or efficiency is estimated by the relative proportion of each metabolite (44). Children were shown to have better methylation capacity than adults in some studies (45-47).

Trivalent compounds are generally more toxic than pentavalent compounds, organic compounds are less toxic than inorganic ones, and the toxicity decreases with increasing methylation. Several *in vitro* and *in vivo* studies have exhibited that MMAIII is the most toxic

arsenical (44, 48, 49). The lower rate of accumulation of pentavalent species is the reason for the much lower toxicity of these species compared to the trivalent compounds (48, 50).

1.1.6 Toxicity and health effects

Arsenic compounds are identified as very toxic and carcinogenic. The most cases of arsenic-induced toxicity in humans are due to exposure to iAs (51). Its toxicity depends on dose and duration of exposure. In humans it can cause death at a single oral dose of 2 mg arsenic/kg and higher. A dose of 0.05 mg arsenic/kg/day over longer periods (weeks to months) can lead to gastrointestinal, hematological, hepatic, dermal, and neurological effects. Whereas, long-term arsenic exposure (years) at a level of 0.001 mg arsenic/kg/day has been linked to skin, bladder, kidney, lung and liver cancer (19). The International Agency for Research on Cancer (IARC) defines arsenic as a Group I known human carcinogen which also induces a wide range of other noncancer effects including non-communicable diseases (NCD) (e.g. cardiovascular diseases, diabetes, anemia, reproductive, developmental, respiratory, immunological, and neurological effects), leaving essentially no human organ system free from potential harm (18, 19, 26, 52). Therefore, these wide array of health problems range from gastrointestinal disturbances to the development of life-threatening conditions like cancer of the kidney, liver, skin, bladder, prostate and lung (16, 53-55).

Exposure to elevated levels of arsenic in drinking water during pregnancy may increase the risk of infant mortality, stillbirth, spontaneous abortion, preterm birth, and low birth weight (44, 56-59) because iAs and its methylated metabolites readily cross the placenta (60). Moreover, early-life arsenic exposure can impair cognitive development in pre-school-aged children (61). Children are mostly susceptible to arsenic poisoning and likely to have more adverse effects than adults (31).

1.1.6.1 Mechanisms of arsenic toxicity

The exact molecular and cellular mechanisms involved in arsenic toxicity are somewhat remain elusive. Arsenic carcinogenicity acts through epigenetic mechanisms rather than as a classical mutagen (62). The mechanisms of arsenic toxicity include the ability of its species to generate reactive oxygen species (ROS) initiating oxidative stress and their differing capacity to cause DNA methylation (both hypomethylation and hypermethylation via interaction with DNA methylating enzymes or by depleting SAM) (63). Thioredoxins, which are antioxidants, can be significantly oxidized by arsenic and that may lead to the generation of ROS (64). The toxicity of different arsenic species can also be attributed to the fact that trivalent arsenicals can bind to the thiol (-SH) groups of proteins and enzymes resulting in the disruption of their functions (65), such as DNA repair enzymes (66) and antioxidant related enzymes, e.g., glutathione peroxidase and thioredoxin reductase (67). Arsenic also shows its toxicity and genotoxicity by different mechanisms like chromosomal aberrations, micronuclei formation, modification of cellular signaling, regenerative cellular proliferation, induction of apoptosis, altered expression and DNA binding activity of transcription factors, and epigenetic modifications (11, 26, 44, 62, 68, 69).

1.1.7 Immune system and its function

The immune system is the body's defense against infectious organisms and other invaders. Through a series of steps called the immune response, the immune system attacks organisms and substances that invade body systems and cause disease. The immune system has two distinct responses, the innate and the adaptive immune responses, which function synergistically to combat against infection. The innate immunity is the first line of defense. It 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 second line of immune defense is the adaptive immunity, which mainly consists of T lymphocytes and B lymphocytes, and their products, such as antibodies. Organs in which lymphocyte precursors mature into antigenically committed, immunocompetent cells and encounter trapped antigens and are activated into effector cells are called lymphoid organs. In mammals, the bone marrow and thymus are the primary lymphoid organ in which B-cell and T-cell maturation occur, respectively. The lymph nodes, spleen, and mucosal-associated lymphoid tissue (MALT) constitute the secondary lymphoid organs. Hematopoietic stem cells (HSC) reside in the bone marrow and give rise to all cell types.

1.1.8 Signal joint T-cell receptor excision circle (sjTREC)

The critical role of the thymus in the generation of a diversified population of peripheral T lymphocytes is well-established. Signal joint T-cell receptor excision circle (sjTREC) is a by-product of T-cell receptor gene rearrangements, generated during lymphocyte maturation in the thymus **(Figure 1)**. They are stable because they lack free DNA ends to be attacked by DNA digesting enzymes, but as they have no origin of replication they do not increase in number when cells divide. Thus, the sjTREC copy number within peripheral T cell populations provides insight into the frequency of recent thymic emigrants or naïve T cells (70).

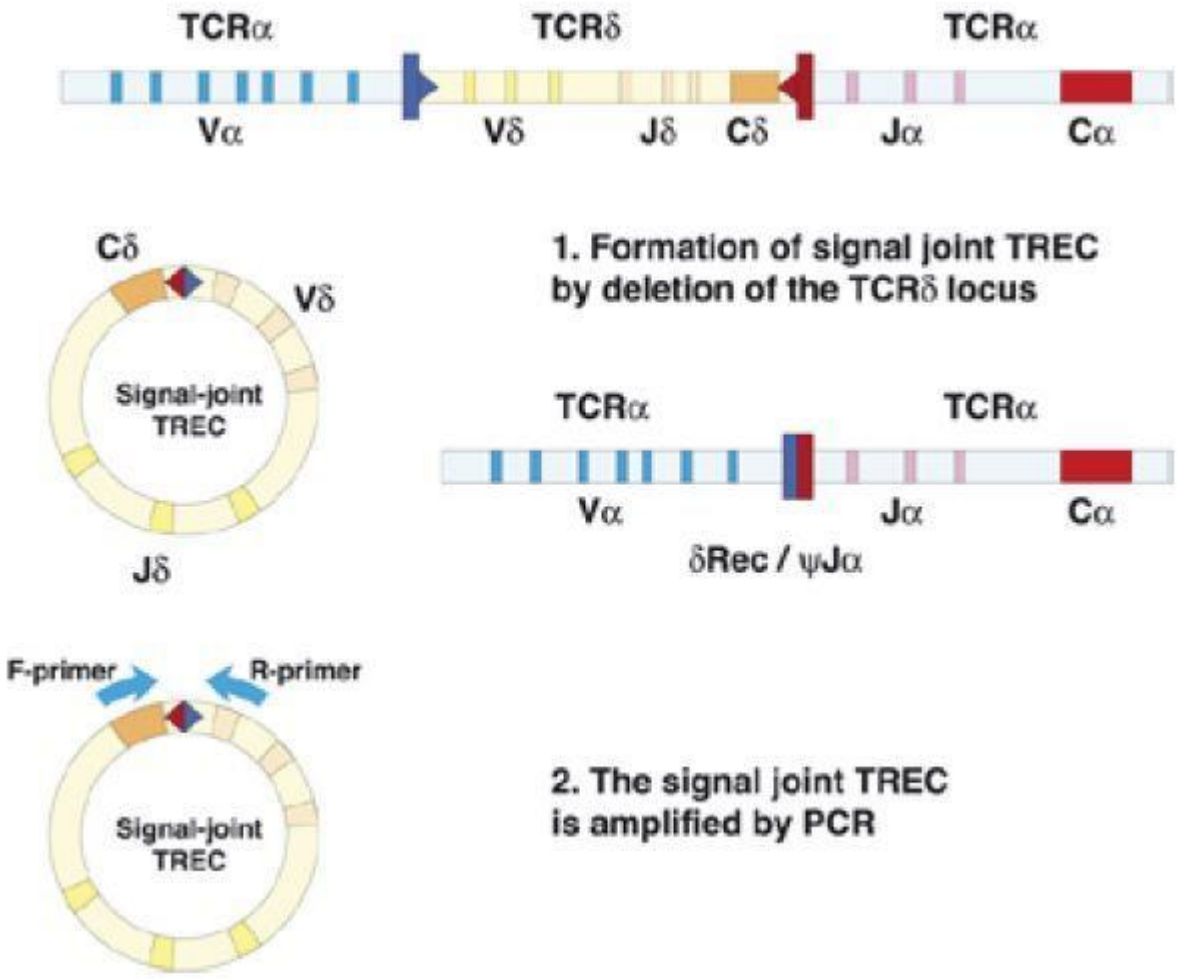

Figure 1: TRECs are circular DNA fragments. They are generated when a section of chromosome 14 (14q11-2), which contains genes responsible for coding T-cell receptors, rearranges. The TRECs are amplified by PCR. (https://newbornscreening.perkinelmer.com/disorders/primary_newborn_screening_disorders/severe_combined_immunodeficiency_(scid))

1.1.8.1 Immunosenescence

Aging of the immune system, or immunosenescence, is defined as a decline in immune function, particularly with respect to lymphocyte number and function (71). Hematopoietic stem cells continually renew cells of the immune system but this capacity declines during senescence which appears to be associated with telomere shortening (72) (discussed in details further below,

section 1.1.11). Any deficiency in the immune response, whether inherited or acquired is termed as immunodeficiency. It can result from defects in phagocytosis, humoral immunity, cell-mediated responses, or some combination of these immune functions.

1.1.9 Immunotoxicity

Immunotoxic effects are often divided into four categories based on the available clinical experience: immunosuppression, immunostimulation, hypersensitivity, and autoimmunity. Each category is associated with relatively specific and clinically distinct adverse effects (73). Immunosuppression is the consequence of an inhibition of the host's immune response. Flu-like reactions, increased incidence of autoimmune diseases, increased incidence of hypersensitivity reactions to varied allergens are characterized by excessive immunostimulation (74, 75). The pronounced immunotoxic effects of drugs and other chemicals in human beings are the consequences of hypersensitivity reactions. A specific adaptive immune response against self-antigens is responsible for autoimmune disease.

1.1.10 Impact of arsenic on immune function

There is adequate evidence for immune dysfunction in mice and humans due to arsenic exposure. Adverse effects of arsenic exposure on the immune system, in particular immunosuppression, were demonstrated by effect on natural T regulatory cells, reduced proliferative response of T cells and IL-2 secretion and apoptosis of immune cells involving adults and children (6−10 years) in few epidemiological studies (76-79). Elevated levels of serum IgG, IgA, and IgE were found in arsenic-exposed adults with skin lesions compared to unexposed adults in Bangladesh (80). Impaired complement function in arsenicosis patients also reveal the immunotoxicity of arsenic (81). A series of studies showed that arsenic-induced immunosuppression occurred as a result of reduced child thymic development and decreased

number of T cells and increased expression of inflammatory cytokines in the placenta (3, 8). They measured sjTRECs as a metric of thymic function (6). This marker was found to be depressed in neonatal cord blood. Reduction of thymic function in these newborns due to prenatal arsenic exposure possibly occurred via induction of oxidative stress and apoptosis of immune cells, indicating subsequent immunosuppression later in life (6). Again, these children at 4.5 years of age, exhibited reduced cell-mediated immunity (DTH response) and Th1 cytokines that were associated with concurrent arsenic exposure (9). Further at 9 years of age, these children showed increased plasma tIgG and tIgE, and impaired mumps vaccine-specific IgG response due to continued arsenic exposure (10).

The immunotoxic effects of arsenic have also been demonstrated in experimental studies (82-84). Lemarie et al. demonstrated that noncytotoxic concentrations of iAs down-regulate differentiation of human primary macrophages which may contribute to immunotoxicity (85). Another *in vitro* study has shown that immune cells of fish leucocytes were specifically vulnerable to the toxic effects of arsenic (86).

1.1.11 Immunosenescence /Telomere length and arsenic

A telomere is a region of nucleotide repeats (TTAGGG) present at each end of the eukaryotic chromosome which is highly regulated to protect chromosomes from recombination or degradation, ensuring integrity during replication. In young humans, telomeres are about 10,000 nucleotides long and lose approximately 50-100 nucleotides per cycle of DNA replication (87). Telomeres shorten after each replication cycle, hence telomere length (TL) is generally considered to reflect cellular senescence (88). The enzyme telomerase (TERT) maintains the TL by adding telomere repeat sequence to the 3′ end of telomeres. Many genetic and environmental factors can affect TL such as oxidative stress, diet, smoking, infections and different types of cancer and non-

communicable diseases (89-91). Shorter and longer TL both are associated with risk of cancer (92, 93). Limited studies have addressed the association of arsenic with TL of human immune cells (94-96). Here we have discussed the accelerated senescence of immune cells due to chronic arsenic toxicity.

1.2 Anemia and Micronutrient Deficiency in Children

Anemia is a condition that develops when there is a deficiency of erythrocytes (red blood cells) or the oxygen-transporting ability of blood cells has been disrupted and hence unable to meet the physiological requirements of the body (97). It is a public health problem affecting about 1.6 billion people worldwide in both poor and affluent countries (97, 98) with infants and pregnant women at particularly high risk of impaired cognitive and physical development (52, 99). Iron deficiency is believed to be the most common cause of anemia (100), however, zinc, folate, vitamin B_{12} and vitamin A deficiencies, chronic inflammation, parasitic infections, and inherited disorders are also potential contributors (101). Ferritin is the most important indicator of the iron status even in the first stage of iron deficiency and iron deficiency anemia (IDA) is diagnosed by measuring serum ferritin concentration (102). However, ferritin is elevated in response to inflammation as it is an acute-phase protein (103). Soluble transferrin receptor (sTfR) is commonly used in the detection of IDA where infection and inflammation are predominant (104), as sTfR level is less affected by inflammation (105). Hepcidin plays a primary role in ensuring the maintenance of an optimal iron store and is used in the diagnosis of IDA and anemia of inflammation (106), in combination with existing diagnostic methods.

Children are particularly susceptible to anemia and micronutrient deficiencies due to the rapid development and growth during this period. Iron deficiency anemia is linked with delayed motor- and neurodevelopment in children (107, 108). The frequency of iron deficiency and IDA

is higher with zinc deficiency in patients including children (109). Reduced immune function and impaired growth and development are also the cause of zinc deficiency in infancy (110, 111). Folate deficiency can lead to impaired synthesis of red blood cells causing megaloblastic anemia (112, 113). Children with folate deficiency have higher risk for acute lower respiratory morbidity and their immune system can also be affected (114). Lack of vitamin B_{12} can also cause megaloblastic anemia (112). Neurological development of children might be affected by vitamin B_{12} deficiency (115, 116). Anemia is associated with low vitamin A status since it has a positive effect on the growth and differentiation of precursor cells of red blood cells and through its effect on iron metabolism or through increased morbidity (117). Vitamin A deficiency leads to impaired immune function and child mortality (110, 118).

The prevalence of anemia in the non-pregnant non-lactating (NPNL) women was 26.0% in the recent Bangladesh National Micronutrients Survey conducted in 2011-12. In this survey, the prevalence rate of anemia was 19.1% in school-age (6-11 years) children (119).

1.2.1 Impact of arsenic on anemia and micronutrients

Considerable evidence supports the observation that arsenic can influence many aspects of the heme system (120-124). Earlier studies have indicated that a suppression of erythropoiesis and direct cytotoxic or hemolytic effect on the blood cells might be the cause of these effects (125, 126). In spite of the high prevalence of anemia mostly in arsenic-contaminated areas (52), few epidemiologic studies have explored the impact of arsenic exposure on anemia. Associations with low-moderate levels of arsenic are even fewer in determining the risk of anemia in pregnant women and children consuming arsenic-contaminated drinking water. There are conflicting reports on the contributing effect of arsenic on anemia. Risk of anemia in association with low to high levels exposure to arsenic via drinking water was observed in arsenic-endemic regions of

Bangladesh and West Bengal, India (121-123). Studies in animals have indicated that iAs can adversely affect hemoglobin (Hb) levels (127). On the other hand, one recent study revealed that prenatal blood arsenic exposure was not a risk factor for the incidence of anemia in pregnant mothers (128). Moreover, no association of anemia was found in all cases of arsenic exposure in different populations (129-131). Breton et al. demonstrated that Hb was significantly associated with arsenic-induced skin lesions only in males but there were no associations between toenail arsenic or urinary arsenic species and Hb levels (132).

Arsenic toxicity to erythrocytes has not yet been assessed in epidemiological studies. A change in the shape or deformability of the cell had been recognized as the toxicity to erythrocytes (133-138). Experimental study revealed that inorganic arsenite can bind to cysteine residues of Hb in rats and humans (139). The trivalent dimethylated arsenical DMA^{III} has shown high affinity to Hb in rat RBCs (140). Another study identified the major arsenic-binding protein (As-BP) and suggested that the DMA^{III}-Hb complex binds to haptoglobin (Hp) to form the ternary DMA^{III}-Hb-Hp complex (141).

Micronutrients can affect absorption, excretion, transport, binding to target proteins, metabolism and sequestration of toxic metals in the body (142-144). Folate deficiency is appeared to decline arsenic methylation capacity significantly and thus enhances arsenic-induced toxicity and apoptosis (145). One comparative study between two different populations exposed to similar arsenic levels revealed variation in the toxicity due to the deficiency of vitamin A (146). It was demonstrated that zinc pretreatment was associated with increased elimination of arsenic (147).

Despite the strong association between arsenic exposure and a range of child health concerns, the mechanisms by which arsenic elicits these effects remain elusive. The information

presented above indicate that pregnant women and their offspring are sensitive groups for arsenic exposure and more studies should focus on the arsenic exposure and associated toxicity in these populations. Therefore, the preceding background has established the rationale for the epidemiologic investigations in the forthcoming dissertation.

1.3 AIMS OF THE THESIS

Overall, there are very few studies suggesting adverse effects of arsenic on immunity and anemia in adults with little or no information available on prospectively collected data on arsenic exposure and other factors starting in early pregnancy. Therefore, in this study in rural Bangladesh children born in our prospective mother-child cohort (the MINIMat cohort, described in Materials & Methods section) were followed-up (9, 148) at 9 years of age, with the aim to examine potential impacts of prenatal and childhood arsenic exposure on immune function and anemia.

Additionally, the research attempts to evaluate whether maternal micronutrient supplementation during pregnancy influences nutritional and micronutrient status in school-age children (~9 years) in a longitudinal mother-child cohort in rural Bangladesh. This study also made an assessment of whether anemia during pregnancy has a long-term impact on anemia in pre-adolescent school-age children and whether this is influenced by maternal micronutrient supplementation.

The two major objectives addressed in the present thesis were to elucidate the effects of prenatal and concurrent arsenic exposure on

(i) anemia related biomarkers (hemoglobin, soluble transferrin receptor, ferritin, vitamin B_{12}, folate, vitamin A, hepcidin, zinc) **(Paper-I)** and

(ii) expression of thymus derived naïve T cells (signal joint T cell receptor excision circles (sjTRECs)) and senescence (telomere length) of immune cells in 9 years old children **(Paper-II)**.

CHAPTER 2
MATERIALS AND METHODS

2.1 Study setting

This thesis builds on data from the study "Maternal and Infant Nutrition Interventions in Matlab" (MINIMat, ISRCTN16581394) carried out in Bangladesh. This trial was conducted in Matlab **(Figure 2)**, a rural subdistrict 57 km south-east of the capital Dhaka where the population receives health services from the International Center for Diarrheal Disease Research, Bangladesh (icddr,b). Here, icddr,b has been operating a health and demographic surveillance system (HDSS) with a central hospital and four smaller regional health care centers, covering a population of about 220,000 in more than 140 villages since 1966. The HDSS database is updated based on information collected through monthly home visits by community health research workers, who record all vital events such as deaths, marriages, in- and out-migrations, pregnancies, pregnancy outcomes, and selected child and maternal morbidity events. Matlab is one of the areas that are most affected by arsenic in drinking water from tube wells in Bangladesh. More than 95% of the population uses tube-well water as their drinking water (149), with 30% having arsenic concentrations above 200 µg/l (150, 151). Recent survey (2013-2014) showed 43% of tube wells in Matlab still contain arsenic >10 µg/L, the WHO guideline value for arsenic in drinking water and 34% tubewells exceeded the 50 µg/L, the national standard for drinking water (152). Agriculture is a major source of income in this area. Therefore, there is substantial exposure to arsenic through food (mainly rice) that comes from the use of arsenic-rich irrigation water from shallow pumps (33).

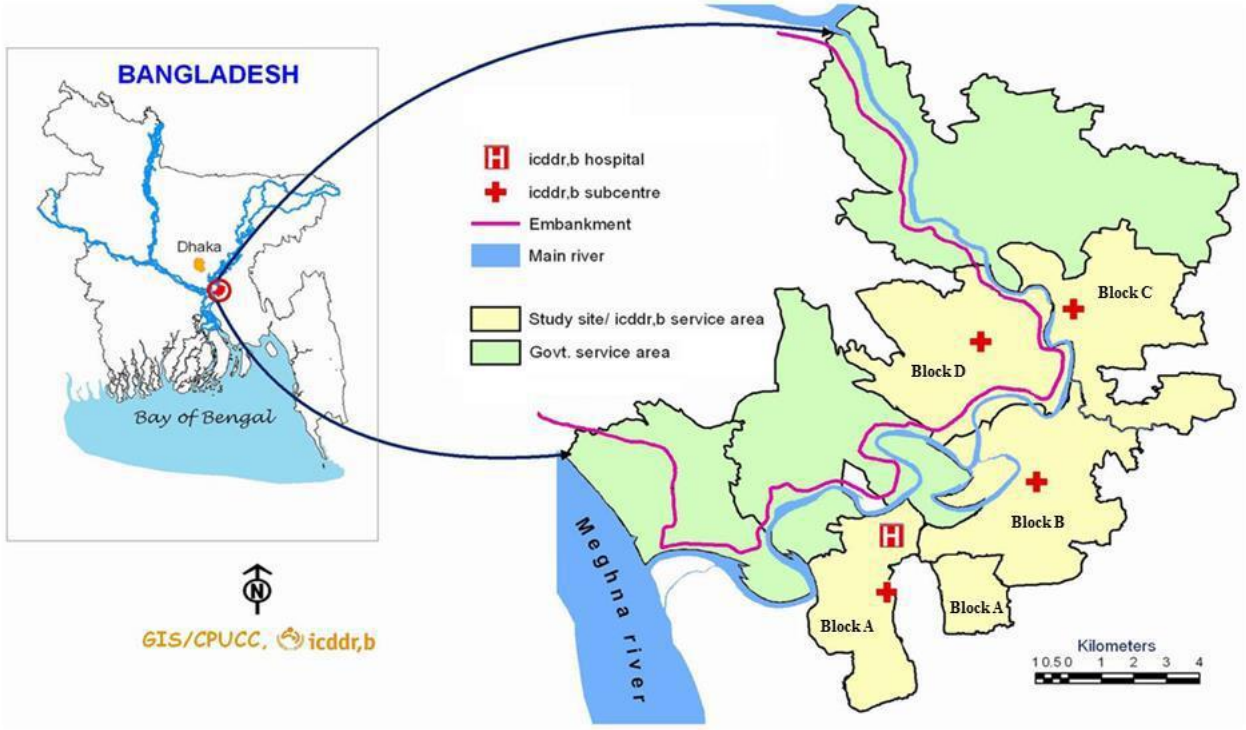

Figure 2: Map of Bangladesh and Matlab study area [adapted from (153) with minor modifications].

2.2 Study design and population

The MINIMat study evaluated the effects of nutritional intervention during pregnancy on birth outcomes, child mortality and growth. Add-on studies further investigated effects of arsenic and other contaminants on maternal and child health including immune function and development. The present thesis is based on data from pregnant women enrolled in the MINIMat trial, and children who were born to mothers in the MINIMat trial and thereafter followed-up at 4.5 and 9 years of age. As part of the HDSS activities, community health workers perform monthly household visits and women were questioned about their last menstrual period and offered a urine pregnancy test if believed to be pregnant. All women identified as pregnant between November 2001 and October 2003 were invited to participate in the MINIMat-trial. Women who gave their

consent were enrolled if not more than 14 weeks pregnant as determined by their last menstrual cycle. The pregnancy was later confirmed by ultrasound. A total of 4436 women were enrolled in the MINIMat trial around gestational week (GW) 8. In total, 3591 women had singleton live birth with anthropometry measurements at birth. The initial urine samples were collected around GW8 for analysis of arsenic concentration. MINIMat was a randomized factorial experiment, where pregnant women irrespective of their nutritional status were randomized to early (E, at about 9 weeks of pregnancy), or usual invitation (U, at about 20 weeks of pregnancy) to daily (6 days a week) food supplement and to one of the three types of micronutrient capsules, (i) 30 mg iron (Fe) and 400 μg folic acid (F) (Fe30F group), or (ii) 60 mg iron and 400 μg folic acid, (Fe60F) or (iii) the UNICEF preparation of multiple micronutrients (MM) including 30 mg iron and 400 μg folic acid (MM group) (154). The MM group contained 15 different micronutrients (vitamins A, D, E, B_1, B_2, B_6, B_{12}, C, Niacin, Folic Acid and minerals Fe, Zn, Cu, I, Se) at the recommended daily allowance level, except folic acid, which was included at level of 400 μg.

For the purpose of study conducted in **Paper-I** and **II**, (155, 156) we only considered women in the MINIMat trial (November 2001 to October 2003) who gave singleton birth in the Matlab hospital or any of the four connected health care facilities from June 2003 to June 2004. From these women, we followed 640 mother–child pairs for studying the effects of arsenic exposure on different biomarkers related to child health and development in children at 4.5 years, and later at 9 years of age as elaborated below.

A total of n = 2735 MINIMat children were studied at the age of 4.5 years (157, 158). To reduce the burden of various types of investigations, the children were divided into two groups on the basis of the calendar year of birth. Group A children (n = 1432) were born between April 2002 to May 2003 and Group B children (n = 1303) were born between June 2003 to June 2004. Group

B children were selected and earlier involved in various studies for asthma, allergy (total group B children, n = 1303, referred in Hawlader et al) (159, 160) as well as immune function and bone growth studies (n = 640 group B children reported in Ahmed et al) from birth and at the age of 4.5 years (3, 6, 8, 9, 161, 162) and followed up at 9 years **(Figure 3)**. The present thesis includes a sub-set of children belonging to Group B. For the present study, field workers carried out a survey for availability of 9 years old children (n=640) by visiting their household who were earlier studied at 4.5 years of age (9). Among these children 39 refused to participate in the follow-up study, 42 migrated, one child was dead, 7 were not available for other reasons, 7 refused to give blood and 4 blood samples were clotted. Finally, 540 blood samples were available for analysis **(Paper I)**. Adequate blood cells for studying sjTRECs and TL were available from 275 children at 4.5 years and 351 children at 9 years of age, and among them 213 children were common in both time points **(Figure 3) (Paper II)**.

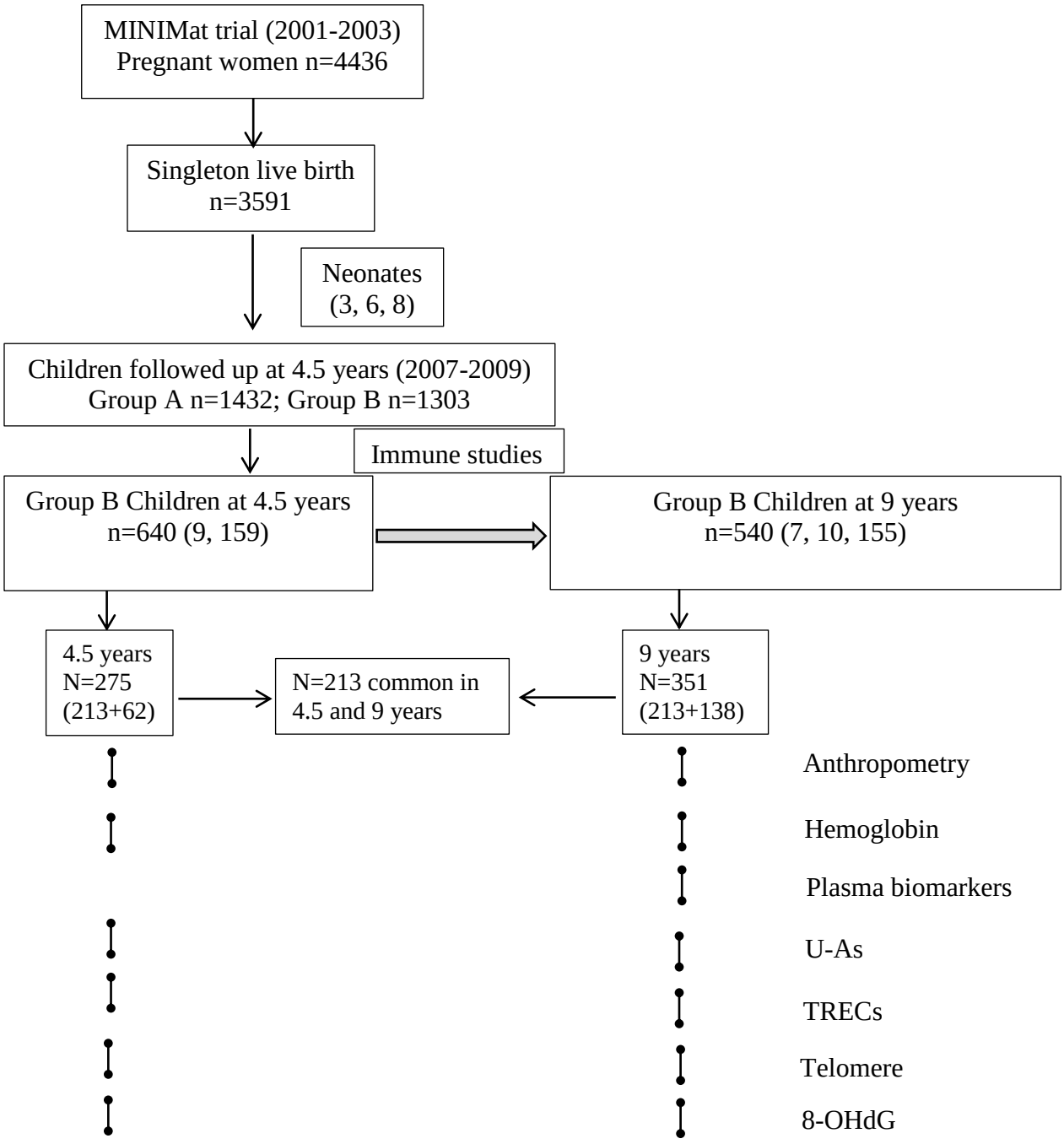

Figure 3: Flow diagram depicting the recruitment of children in the study nested in the MINIMat birth cohort study in rural Bangladesh carried out in 2012–2013.

2.3 Data Collection and Sampling

Maternal anthropometry and family socioeconomic status were collected in early pregnancy in the MINIMat trial (154). Baseline data for mothers were taken by trained health research workers at the first sub-center visit in pregnancy week 8. The study interviewers visited all participating women and registered pregnancy outcomes (spontaneous abortion, induced abortion, stillbirth, and live birth). Anthropometry data of children were collected at 4.5 and 9 years (10). Anthropometric measurements were taken at the sub-center clinic visit by trained nurses. Weight was measured with a digital scale (TANITA HD-318, Tanita Corporation, Japan), accurate to ±10g. Weight scales were standardized daily. Height was measured using a free-standing stadiometer Leicester Height Measure with millimeter marks (Seca214, UK). The stadiometer was calibrated before the start of the study and in every 6 months. The measured weight and height were converted to weight-for-age (WAZ), height-for-age (HAZ) and body mass index (BMI)-for-age Z-scores (BAZ) (standard deviation (SD) scores), using the WHO Multicentre Growth Reference Study child growth standards for school-aged children and adolescents (163). Children with WAZ <-2 SD from WHO reference population were considered as underweight, with HAZ <-2SD as stunted, and those with BAZ <-2SD were considered as thin.

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.

Socioeconomic status (SES) of the families was retrieved from the HDSS databases at the enrollment in the MINIMat trial. It 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. The family SES score was estimated via an asset index, generated through principal component analysis of household assets and grouped into tertiles (164). It was further updated during the follow-up studies.

Five ml of fasting venous blood was collected in Lithium heparin treated tubes (Sarstedt Monevette®, Sweden) by trained paramedics in the subcenters in Matlab. Heparinized blood was centrifuged to separate plasma from buffy coat. Peripheral blood mononuclear cells (PBMC) was separated from buffy coat by density-gradient centrifugation and stored in RNALater (Qiagen GmbH, Hilden, Germany) in -80°C. Urine samples from pregnant mothers during GW8 and their children at 4.5 and 9 years of age 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 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 -80°C) 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.

2.4 LABORATORY ANALYSES

2.4.1 Assessment of plasma biomarkers

A drop of blood from mothers (at GW14 and GW30) and children of 4.5 and 9 years was used to measure Hb by HemoCueHb 201+ (HemoCue AB, Ängelholm, Sweden). The blood samples were transported to the Matlab Laboratory for separation of plasma which was thereafter stored in -80°C freezer until transported to the Laboratory in icddr,b, Dhaka. Ferritin, vitamin B_{12} and folate were analyzed in plasma by chemiluminescence method using Cobas e601 (Roche Diagnostics, Mannheim, Germany). C-reactive protein (CRP) and soluble transferrin receptor (sTfR) were assessed in plasma using Hitachi 902 (Roche Diagnostics, Mannheim, Germany). Plasma hepcidin was measured using a commercial ELISA kit (DRG International, GmbH, Germany). Flame atomic absorption spectroscopy was used to analyze plasma zinc (Zn) concentrations (Shimadzu Corporation, Kyoto, Japan). Isocratic reverse-phase HPLC and UV detection were used to analyze concentrations of plasma retinol/vitamin A (Shimadzu Corporation, Kyoto, Japan). The mean inter-day coefficient of variation was <5% for sTfR, ferritin, folate, vitamin B_{12}, Zn, vitamin A, and CRP and <8% for hepcidin. For an independent assessment of the laboratory's analytical performance, it participates in external quality assurance programs such as VITAL EQA of Centers for Disease Control and Prevention (CDC) for the above parameters excepting Zn.

The complete blood count (CBC) including mean corpuscular volume (MCV), mean corpuscular Hb (MCH), mean corpuscular Hb concentration (MCHC) and other blood indices were determined using Hematology Analyzer (Sysmex XT-1800i, Kobe, Japan). Erythrocyte Sedimentation Rate (ESR) was measured by Wintrobe Method (NCCLS) **(Paper I)**.

2.4.1.1 Hemoglobin and anemia

Anemia is defined as Hb<115 g/l for 9 years and <110 g/l for <4.5 years old children according to WHO guidelines (12) **(Table 5)**. Mild anemia is defined if Hb level is 110-114 g/l, moderate is 80-109 g/l, and severe anemia is <80 g/l (12). The cut-off to define anemia in pregnancy was set to Hb<110 g/l according to (52). The cut-off values to define iron deficiency (ID), iron deficiency anemia (IDA) (12, 165), and micronutrient deficiencies (100, 166, 167) have been described in **Table 5**.

2.4.2 Urinary arsenic

Arsenic exposure was assessed based on the concentration of the sum of iAs, MMA and DMA in urine, hereinafter referred to as urinary arsenic (U-As) reflecting exposure to iAs from both water and food (33, 168). The arsenic metabolite concentrations in urine were measured using high-performance liquid chromatography online with hydride generation and inductively coupled plasma mass spectrometry (HPLC-HG-ICPMS). The intra- and inter-assay coefficients of variation were approximately 4%, based on the measurements of a reference urine sample (CRM No.18, National Institute for Environmental Studies, Tsukuba City, Japan) (9). In order to compensate for variation in dilution in urine samples all U-As concentrations were adjusted to the average specific gravity (1.012 g/ml for all urine samples), measured by a digital refractometer (RD712 Clinical Refractometer; EUROMEX, Arnhem, the Netherlands) using the formula U-As (1.012-1)/(measured specific gravity of each urine sample-1). The difference in dilution of urine is caused by variation in fluid intake as well as differences in physical activity and temperature. 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 (169) **(Paper II)**.

2.4.3 Estimation of sjTRECs

From the frozen PBMC, DNA was isolated by using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. From 200 ng of DNA/sample, quantification of sjTREC was performed by using SYBR Green real-time quantitative PCR and CFX96TM real time system (C1000^{TM} Thermal cycler, Bio-Rad Life Science Research, Hercules, CA) as described earlier (6, 170). The following primers were used: forward primer 5′AAAGAGGGCAGCCCTCTCCAAGGCAAA3′ and reverse primer 5′AGGCT-GATCTTGTCTGACATTTGCTCCG3′. A standard (kindly donated by PT Ngom) was prepared by using serial dilutions of a known number of copies of a fragment of the sjTREC gene sequence and included in each run to generate a standard curve (171). A master mix was prepared that consisted of 10.0 µL SYBR Green (Qiagen), 1.0 µL forward and reverse primers each, and 6.0 µL deionized water. Then, 18 µL of this master mix was added to the 96-well plate, which was followed by 2 µL of standards, samples, and negative controls in corresponding wells to obtain a 20 µL reaction volume. Real-time PCR was performed under the following conditions: denaturation (one cycle) at 95°C for 3 min; preamplification of 40–45 cycles at 95°C for 30 sec, 62°C for 30 sec, 72°C for 45 sec; and amplification (final extension step) cycle at 72°C for 5 min. The copy number of sjTRECs in the DNA samples was determined automatically using standard curves and expressed as sjTRECs content (copy numbers/100 ng of DNA) **(Paper II)**. The coefficient of variations (CV%) of intra- and inter-run variation of determination of sjTREC level were 8.5% and 10.2% respectively.

2.4.4 Estimation of Telomere length

Absolute TL in PBMC DNA was measured based on method reported by O'Callaghan & Fenech (172) by performing two RT-PCR reactions using 70 ng of DNA samples for each reaction in a single plate using two different oligomer standards. Telomere oligomer standard was used to establish telomere standard curve in telomere PCR reaction whereas single copy gene 36B4 oligomer standard was used for creating 36B4 standard curve in 36B4 PCR reaction. The primer sequences were: telomere forward primer 5'CGGTTTGTTTGGGTTTGGGTTT-GGGTTTGGG-TTTGGGTT3'; and reverse primer 5'GGCTTGCCTTACCCTTACCCTTACCC-TTACCC-TTACCCT3'; 36B4 forward primer 5'CAGCAAGTGGGAA-GGTGTAATCC3' and reverse primer 5'CCCATTCTATCATC-AACGGGTACAA3'. A master mix was prepared that consisted of 10.0 µL SYBR Green (Qiagen), 1.0 µL forward and reverse primers each, and 6.0 µL deionized water. Then, 18 µL of this master mix was added to the 96-well plate, which was followed by 1 µL of standards, 1 µL of plasmid DNA, 2 µL of samples, and negative controls in corresponding wells to obtain a 20 µL reaction volume. Plasmid DNA (pBR322) was used with each standard to maintain a constant 20 ng of total DNA per reaction tube. Real-time PCR was performed as described in section 3.4.3. The data obtained from RT-PCR method to measure absolute TL were analyzed as kb/reaction and genome copies/reaction for telomere and 36B4, respectively. The telomere kb per reaction value is divided by diploid genome copy number of 36B4 to give a total TL in kb per human diploid genome **(Paper II)**. The C_T values within the linear range of the standard curves and between 15 and 35 threshold cycles were used for analysis. The CV% of intra- and inter-run variation of TL assessment were <5% and <7.5% respectively.

2.4.5 Oxidative stress marker

Arsenic is known to produce reactive oxygen species leading to oxidative stress and DNA damage (173). This can be assessed by determining 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentration, a known biomarker of oxidative DNA damage. Plasma concentration of 8-OHdG was analyzed by a competitive ELISA kit (Highly Sensitive 8-OHdG Check ELISA, Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan) **(Paper II)**. Samples were measured in duplicates and analysis was repeated if results of duplicate samples differed more than 10%. The intra- and inter- assay coefficient of variations were 2.85% and 4.6% respectively.

2.5 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.

2.6 STATISTICAL ANALYSES

Statistical analyses were conducted using the software PASW 20.0 (SPSS Inc., Chicago, IL) and Stata/IC 13.0 (StataCorp, College Station, Texas, USA). Normality (data distribution patterns) and homogeneity of variances were formally checked. Associations between exposures, outcomes, and covariates were initially evaluated using Spearman's rank correlation (for

continuous variables), Mann-Whitney U-test, analysis of variance, or Kruskal-Wallis test (for categorical variables), as appropriate.

Associations between different exposure biomarkers (U-As at 4.5 and 9 years; MMA%, DMA%, iAs%) and outcomes (sjTRECs and TL) were examined graphically using lowess moving average curves searching for linear or non-linear associations. When linear associations were obtained, multivariable-adjusted linear regression analyses were carried out. When deviation from linearity was found, we applied splines at apparent thresholds. In scatter plots with Lowess curves (**Figure 4A-F**), both TL and sjTRECs slightly increased up to 45 µg/L of U-As and then started to decline showing deviation from a linear pattern. Thus, due to the nature of non-linear curve the associations of U-As at all-time points (GW8, 4.5 and 9 years of age) with TL and sjTRECs were assessed by multivariable-adjusted spline regression analyses with a spline knot introduced at 45 µg/L (log_2-transformed: equivalent to 5.5). To obtain normally distributed residuals with a homogeneous variance, all exposure variables (U-As at GW8, 4.5 and 9 years of age) were log_2-transformed. We chose log_2-transformation of TL and sjTRECs values to simplify the interpretation of the beta-coefficients in the regression analyses (average changes in outcome associated with each doubling of exposure). To analyze the influence of arsenic metabolism efficiency on the associations between U-As and sjTRECs or TL, initially we analyzed by multivariable-adjusted linear regression. Again, we observed non-linear association between % of arsenic metabolites and telomere length or sjTRECs, thus spline regression was applied. The spline knot values of the metabolites were introduced at 7, 80 and 15 for MMA%, DMA%, iAs% respectively. Linear mixed effects models were applied to evaluate the effect of exposure over time (4.5 and 9 years of age) on outcome changes (TL and sjTRECs) with a spline knot at 45 µg/L. The association between U-As and 8-OHdG was evaluated by linear regression analysis.

Analysis of covariance with least significant difference (LSD) was used for multiple comparisons of child nutritional status and plasma biomarkers in relation to supplementation groups. Linear regression analyses were used to evaluate the influence of plasma biomarkers on Hb levels. Multivariable adjusted logistic regression analyses were used to evaluate the risk of having anemia in 9 years old children if they were anemic at 4.5 years of age or their mothers were anemic during pregnancy.

Statistical models were adjusted for covariates that were significantly associated with both exposure and outcome, or biologically relevant or changed the effect estimates by 5% or more. The covariates were child gender, age, BMI, HAZ, SES, mother's education, mother's occupation and plasma CRP. P values <0.05 were considered statistically significant.

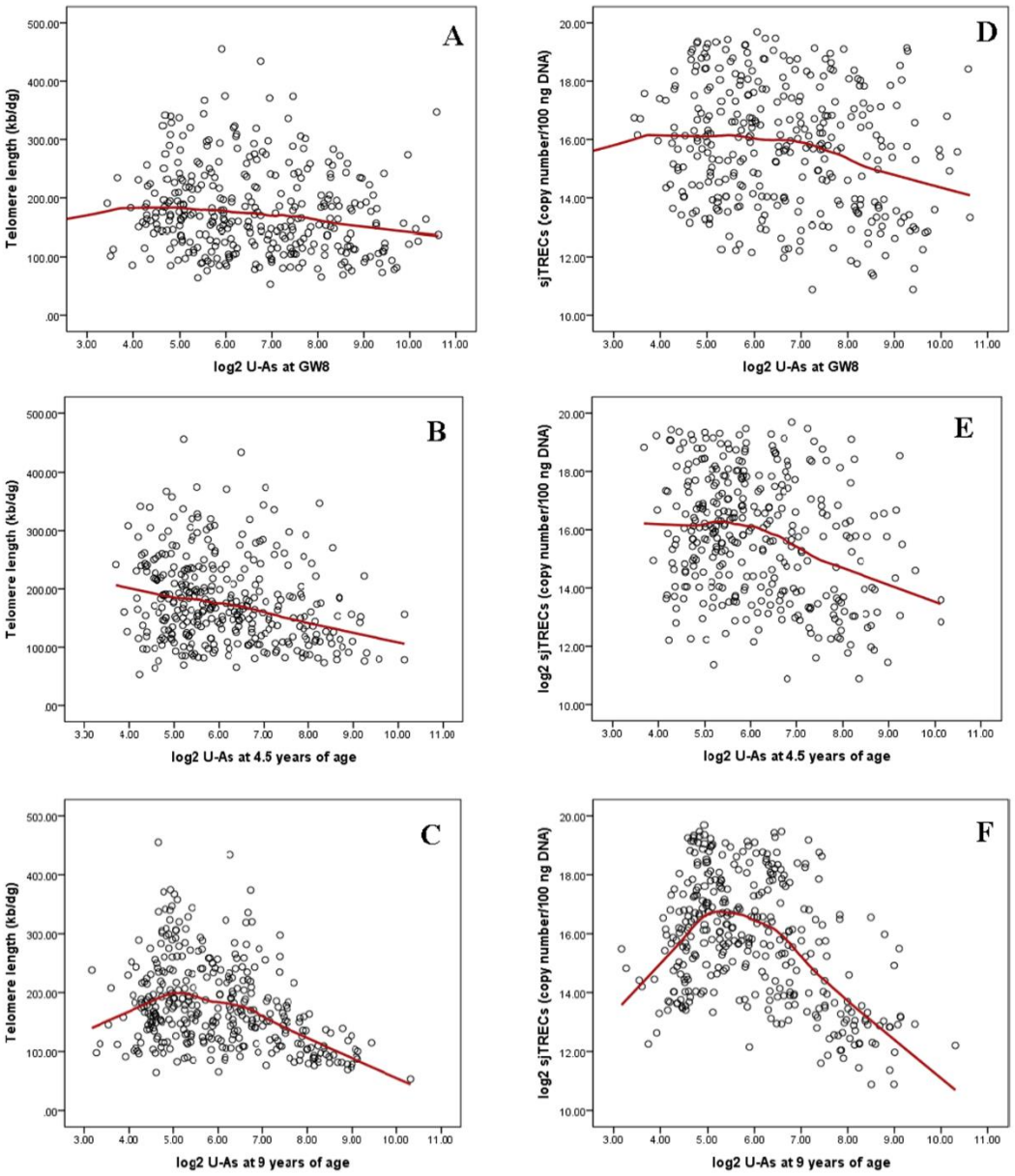

Figure 4: Associations between urinary arsenic concentration (U-As) (log_2-transformed) at GW8, 4.5 and 9 years and telomere length (A, B and C) and signal joint T cell receptor excision circles (sjTRECs) (log_2-transformed) (D, E and F) in 9 years old children. In the scatter plots, the solid line represents a Lowess (locally weighted scatter plot smoothing) moving-average curve for the raw data.

CHAPTER 3
RESULTS

3.1 General characteristics of women and children

The sample size in the two papers varies as not all biomarkers were assessed for all individuals, however, all women were randomly selected for use in different subsets (please also see description in page 19, under 2.2, **Figure 3**). Basic characteristics of the children were presented in **Table 1**. The median age of the children at the different follow-ups were 4.6 years (range: 4.5-5.2) and 8.8 years (range: 8.6-9.6), respectively and were referred to as 4.5 and 9 years of age. At 9 years of age, about 22% children were stunted and 40% were underweight. Among the stunted children, 10% were severely stunted (HAZ<-3SD) while among the underweight children, 26% were severely underweight (WAZ<-3SD). About 26% children were thin (**Table 1**); among them 21% were severely thin (BAZ<-3SD).

At 4.5 years of age, about 30% children were stunted, among them, 16% were severely stunted. About 39% children were underweight and among them about 19% were severely underweight. Again, about 13% children were thin, of which 10% were severely thin.

The mean Hb concentration of 9 years old children was 123.81±9.06 g/l (mean±SD) **(Table 1)**. There was no difference in the Hb concentration between boys and girls. Mothers' mean Hb were 116.98±12.70 g/l (mean±SD) and 113.98±11.55 g/l (mean±SD) at GW14 and GW30 respectively.

Median U-As in the children at 9 years was lower than that of their mothers during pregnancy (median 88, range 1.9-1576 µg/L) and at 4.5 years of age. The mother's U-As concentrations were strongly correlated with children's exposures (r_s=0.48 for 4.5 years and r_s=0.40 for 9 years, both p<0.001) suggesting similar level of arsenic exposure in mothers and children. Family SES did not change significantly over time from GW8 to 9 years of age. There was strong association between TL at 9 years with TL at 4.5 years (**Figure 5**), even though the

median TL at 9 years was lower than that at 4.5 years (p<0.001). Mean plasma concentration of 8-OHdG was significantly higher in 9 years-olds compared to 4.5 years-olds (p<0.001) (**Table 1**).

Table 1: Baseline characteristics of the study participants.

Variables[a]	4.5 years (n=213)	9 years (n=351)
Age, Months	55.9 ± 1.4	104.4 ± 1.2
Gender, Male, *n* (%)	99 (46.5%)	178 (50.7%)
Weight (kg)	13.7 ± 1.5	22.0 ± 3.1
Height (cm)	100.0 ± 4.0	123.0 ± 5.2
Stunted[b], *n* (%)	61(28.6%)	76(21.7%)
Underweight[b], *n* (%)	97(45.5%)	147(41.9%)
Thinness[b], *n* (%)	69 (12.78%)	137 (25.50%)
Hb (g/l)	128.61±13.33	123.81±9.06
U-As (µg/L)[c]	57.1 (12.9, 1125.0)	53.9 (8.9, 1268.0)
Telomere length (kb/dg)	188.9 (68.6-464.0)	164.8 (53.3-455.0)
sjTRECs (copy number/100 ng DNA)	4.8 (0.23-684.0)x10^4	5.7 (0.18-84.5)x10^4
8-OHdG (ng/mL)	2.5 ± 1.4	3.3 ± 1.7
Family SES[d]		
1st tertile, *n* (%)	71 (33.3%)	117 (33.3%)
2nd tertile, *n* (%)	65 (30.5%)	117 (33.3%)
3rd tertile, *n* (%)	77 (36.2%)	117 (33.3%)

[a]Values are given either as mean ± SD or median with range within brackets.
[b]Defined as children with HAZ, height for age, WAZ, weight for age or BAZ, body mass index (BMI) for age <-2 SDs from the median value of height, weight or BMI for age of reference population, respectively (according to WHO).
[c]Adjusted to average specific gravity of 1.012.
[d]SES score was estimated via an asset index, generated through principal component analysis of household assets.

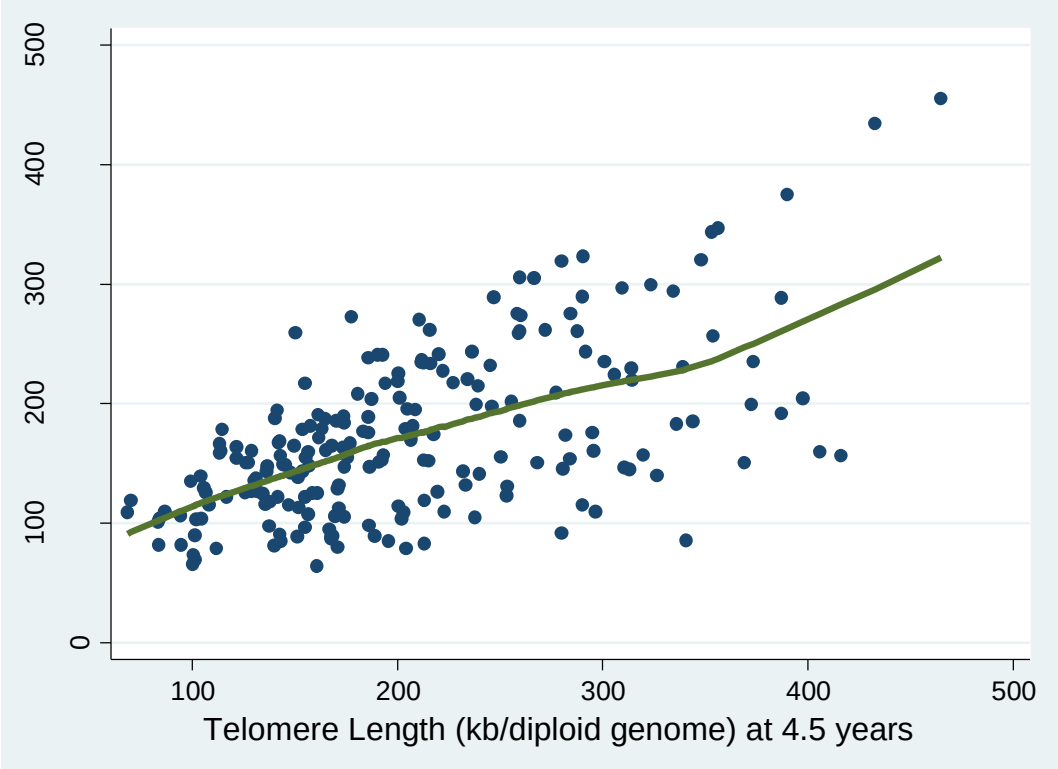

Figure 5: Association of telomere length between 4.5 and 9 years of age (*n*=211). In the scatter plots, the solid line represents a Lowess (locally weighted scatter plot smoothing) moving-average curve for the raw data.

Examinations of the scatter plots (**Figure 4A-F**) of TL and sjTRECs (log_2-transformed) against U-As (log_2-transformed) at GW8, 4.5 and 9 years in 9 years old children indicated clear nonlinear relationships. Both TL and sjTRECs slightly increased up to 45 µg/L of U-As and then started to decline.

3.2 Hb and arsenic exposure

We did not find any association of Hb levels in mothers at GW14 or 30 with U-As at GW8. Also, no association was observed between Hb and U-As in children of 4.5 and 9 years of age **(Table 2)**. Similar outcomes were revealed when the associations between U-As metabolites (%MMA, %DMA and %iAs) and Hb were considered (Data not shown). Association of U-As

with anemia related plasma biomarkers was not found as well. However, folate and zinc showed negative associations with iAs. Zinc also significantly associated with DMA in children of 9 years.

Table 2: Regression analysis of urinary arsenic concentration at gestation week 8, 4.5 and 9 years with hemoglobin at gestation week 14 & 30, 4.5 and 9 years of age.

Hb	U-As at GW8	U-As at 4.5 years	U-As at 9 years
	β (95% CI)[a]	β (95% CI)[a]	β (95% CI)[a]
Mothers at GW14	-0.02(-0.27, 0.21)	0.05(-0.24, 0.35)	-0.02(-0.31, 0.27)
Mothers at GW30	-0.01(-0.23, 0.21)	-0.05(0.33, 0.23)	-0.07(-0.34, 0.19)
Children at 4.5 years		0.01(-0.29, 0.30)	0.14(-0.14, 0.43)
Children at 9 years			1.28(-0.65, 15.4)

[a]Adjusted for child age, height-for-age z-score, gender, plasma C-reactive protein and total leukocytes at 9 years, family SES at 9 years, mothers' education.

3.3 Status of nutritional biomarkers (Paper I)

The prevalence of anemia was about 15% in 9 years and 5% in 4.5 years old children. Among the 9 years old anemic children, 55% had mild, and 45% had moderate anemia. Severe anemia was not present among the children.

Descriptive statistics of all nutritional biomarkers have been given in **Table 3**. Folate deficiency (<5.2 nmol/l) was not observed among the study participants; only 5 children (0.93%) had vitamin B_{12} deficiency (<182 pmol/l). About 13% children had folate (>29.5 nmol/l) and 3.1% had vitamin B_{12} concentration (>867 pmol/l) above the reference range. Zinc and vitamin A deficiencies were present in 8% and 6.2% children respectively. All studied children were hepcidin deficient when 53.5 µg/l was considered as a cut-off, but only 5 children were deficient when the cut-off was <1 µg/l. Iron deficiency was found in 8.0% children when sTfR cut-off was used.

However, it was only 0.2% when ferritin cut-off was used. About 2.5% children had IDA according to the defined sTfR cut-off and 0.2% (one child) when applying the ferritin cut-off **(Table 4)**.

Table 3. Plasma biomarkers in school-age children in rural Matlab.

	All children (n=540)	Fe30F (n=188)	Fe60F (n=185)	MM (n=167)
sTfR (nmol/l)	44.83±10.96	45.34±11.38	44.67±11.61	44.45±9.69
Ferritin (µg/l)	59.82±31.35	62.04±30.10	59.57±28.55	57.59±35.42
Folate (nmol/l)	23.13±5.74	23.78±5.96	22.53±5.64	23.05±5.57
Vitamin B_{12} (pmol/l)	469.96±174.67	474.88±167.78	460.38±179.18	474.95±177.78
Hepcidin (µg/l)	9.57±5.92	9.85±6.65	9.60±5.69	9.23±5.29
Zinc (µmol/l)	13.33±2.54	13.34±2.55	13.36±2.64	13.29±2.43
Vitamin A (µmol/l)	1.03±0.22	1.03±0.22	1.04±0.23	1.01±0.21

Data is presented as mean ± standard deviation.

Based on morphological classification of anemia, about 61% of anemic children (n=50) and 92% of non-anemic children (n=431) had normocytic normochromic RBC. About 34% of anemic children and 6.3% of non-anemic children had microcytic RBC while 4.9% of anemic and 1.3% of non-anemic children had normocytic hypochromic RBC **(Table 4)**.

Table 4. Indicators used in this thesis to define anemia and micronutrient deficiencies in school-age children.

Condition	Indicator	Cut-off
Anemia	Hb	< 115 g/l for 9 years (12) <110 g/l for <4.5 years and in pregnancy (12)
Iron deficiency (ID)	Plasma ferritin or Plasma sTfR	<15 µg/l (12) or >59 nmol/l (165)
Iron deficiency anemia (IDA)	Plasma ferritin and Hb or Plasma sTfR and Hb	<15 µg/l and <115 g/l (12) or >59 nmol/l and <115 g/l (12, 165)
Folate deficiency	Plasma folate	<5.2 nmol/l (166)
Vitamin B_{12} deficiency	Plasma vitamin B_{12}	< 182 pmol/l (166)
Hepcidin deficiency	Plasma hepcidin	<1 µg/l (41) <53.5 µg/l (174)
Zinc deficiency	Plasma zinc	<9.9 µmol/l (167)
Vitamin A deficiency	Plasma vitamin A	<0.7 µmol/l (100)
Normocytic normochromic Anemia	MCV and MCH	74.4-93.9 fl and 24.3-31.8 pg (175)
Microcytic anemia	MCV	<74.4 fl (175)
Normocytic hypochromic anemia	MCV and MCH	74.4-93.9 fl and <24.3 pg (175)

3.4 Influence of maternal supplementation on child growth and inflammatory markers (Paper I)

Compared to the Fe30F group, children in the Fe60F as well as MM group had significantly higher BAZ scores at 9 years of age **(Table 5)**. However, no significant differences were seen in HAZ or WAZ scores with respect to supplementation. Maternal supplementation did not affect the number of stunted or underweight children either at 4.5 or 9 years of age. However, 9 years old

children belonging to the MM and Fe60F groups had fewer thin children (n=36 and 41, respectively) compared to those in Fe30F group (n=60, *p*=0.02, and 0.04, respectively) (Data not shown).

Table 5. Analysis of covariance of child nutritional status and plasma biomarkers in different supplementation groups

	Fe30F (n=188)	Fe60F (n=185)	*p* -value[1]	MM (n=167)	*p* -value[2]
Nutritional status					
HAZ at 4.5 years	-1.53±0.86	-1.55±0.88	0.59	-1.59±0.87	0.54
HAZ at 9 years	-1.27±0.86	-1.27±0.93	0.98	-1.40±0.88	0.19
WAZ at 4.5 years	-1.76±0.84	-1.78±0.83	0.58	-1.76±0.76	0.98
WAZ at 9 years	-1.68±1.04	-1.67±1.11	0.80	-1.74±0.96	0.38
BAZ at 4.5 years	-1.17±0.82	-1.18±0.76	0.97	-1.11±0.78	0.33
BAZ at 9 years	-1.47±1.09	-1.25±1.08	0.05	-1.23±1.06	0.04
Biomarkers					
Hb at 4.5 years	127.76±8.90	129.79±9.49	0.15	128.08±8.77	0.76
Hb at 9 years	123.54±12.50	124.26±13.45	0.52	123.56±14.03	0.98
Folate (nmol/l)	23.66±5.96	22.60±5.64	0.06	20.03±5.57	0.03
ESR (mm/1st hr)	16.15±10.95	15.76±10.43	0.75	14.25±10.28	0.05
CRP (mg/L)	1.36±2.97	0.91±1.60	0.04	0.60±0.63	0.002
RDW-CV (%)	13.67±1.22	13.43±0.96	0.05	13.54±1.05	0.29
MCH (pg)	26.35±2.25	27.50±6.64	0.01	26.64±2.03	0.57

[1]Significant difference between Fe30F and Fe60F
[2]Significant difference between Fe30F and MM
Models were adjusted for SES, body mass index, child gender, mother's occupation, mother's education levels and plasma concentration of C-reactive protein;
Data are presented as mean ± standard deviation.

When markers of inflammation or infection were considered, children in MM group had significantly lower levels of CRP and ESR compared to the Fe30F group **(Table 5)**. Children in the MM group had significantly lower concentration of plasma folate compared to the Fe30F group.

When considering RBC indices, children in Fe60F group had higher MCH concentration compared to Fe30F group. RDW values were significantly lower in the Fe60F group compared to Fe30F group **(Table 5)**.

3.5 Association of Hb with plasma biomarkers and effect of maternal micronutrient supplementation (Paper I)

In the adjusted linear regression analysis, Hb was significantly inversely associated with plasma sTfR; when the association between Hb and sTfR was stratified by gender the association remained significant only in boys **(Table 6)**. Hb was positively associated with hepcidin in all children and in girls only. Hb was also positively associated with vitamin A in all children **(Table 6)**. A positive tendency was obtained between Hb and Zn in girls, and a positive association was noted between Hb and vitamin B_{12} in boys only.

Table 6. Linear regression analyses between hemoglobin concentrations and plasma biomarkers in all children and boys and girls separately.

Variables	All supplementation groups					
	All children (n=540)		Boys (n=263)		Girls (n=277)	
	*Adj. β (95% CI)	*P*	#Adj. β (95% CI)	*p*	#Adj. β (95% CI)	*p*
sTfR (nmol/l)	-0.75(-1.48, 0.01)	0.04	-1.46(-2.38, -0.55)	0.002	0.46(-0.78, 1.70)	0.46
Ferritin (µg/l)	0.13(-0.12, 0.38)	0.31	0.20(-0.15, 0.56)	0.26	0.06(-0.30, 0.43)	0.73
Folate (nmol/l)	-0.11(-1.47, 1.26)	0.87	-1.01(-2.98, 0.96)	0.31	1.03(-0.92, 3.00)	0.30
Vitamin B_{12} (pmol/l)	0.02(-0.02, 0.06)	0.39	0.07(-0.001, 0.14)	0.05	-0.01(-0.07, 0.05)	0.64
Hepcidin (µg/l)	1.274(0.26, 3.31)	0.02	1.12(-1.44, 3.69)	0.38	1.92(0.09, 3.75)	0.04
Zinc (µmol/l)	1.65(-1.44, 4.73)	0.29	-0.85(-5.20, 3.50)	0.70	3.98(-0.48, 8.44)	0.08
Vitamin A (µmol/l)	2.16(0.88, 3.45)	0.001	3.02(1.04, 5.00)	0.003	1.70(0.01, 3.38)	0.04
	Fe60F					
	All children (n=185)		Boys (n=87)		Girls (n=98)	
sTfR (nmol/l)	-0.79(-2.06, 0.48)	0.22	-1.06(-2.74, 0.62)	0.21	-0.5(-3.03, 1.53)	0.51
Ferritin (µg/l)	0.47(-0.02, 0.97)	0.06	0. 72(-0.10, 1.53)	0.08	0.37(-0.27, 1.01)	0.25
Folate (nmol/l)	0.32(-2.22, 2.86)	0.80	-1.06(-4.84, 2.72)	0.57	3.12(-0.72, 6.96)	0.11
Vitamin B_{12} (pmol/l)	0.04(-0.04, 0.12)	0.27	0.19 (0.04, 0.34)	0.01	-0.02(-0.11, 0.07)	0.66
Hepcidin (µg/l)	2.88(0.35, 5.41)	0.02	0.80(-4.31, 5.91)	0.75	4.11(1.20, 7.02)	0.006
Zinc (µmol/l)	-1.05(-6.57, 4.47)	0.70	-5.25(-13.57, 3.07)	0.21	4.29(-4.00, 12.57)	0.30
Vitamin A (µmol/l)	3.13(0.89, 5.38)	0.006	6.04(2.44, 9.64)	0.001	1.11(-1.84, 4.06)	0.45

Data were given as regression coefficient (β) and 95% confidence intervals;
*Adjusted for SES, body mass index, child gender, mother's occupation, mother's education levels and plasma concentration of C-reactive protein;
#Adjusted for SES, body mass index, mother's occupation, mother's education levels and plasma concentration of C-reactive protein.

To evaluate the influence of maternal supplementation, the associations between Hb and plasma biomarkers in 9 years old children were stratified by different supplementation groups **(Table 6)**. In the Fe60F group but not others (Fe30F and MM, data not shown), Hb was strongly positively associated with plasma hepcidin and vitamin A in all children. When these associations were stratified by gender, Hb was associated with vitamin A in boys and with hepcidin in girls **(Table 6)**. Again, in Fe60F group, the significant positive association was noted between Hb and vitamin B_{12} only in boys **(Table 6)**.

3.6 Long term impact of maternal anemia in school-age children (Paper I)

Among mothers during pregnancy, about 28% and 35% of the women at GW14 and GW30, respectively, were found to be anemic, and 23.6% women were anemic at both time points. Percentage of anemia was higher in children at 9 years of age (15%) compared to 4.5 years of age (5%). Notably, thirteen children who were anemic at 4.5 years of age (45%) remained anemic at 9 years of age. Sixty nine children who were non-anemic at 4.5 years became anemic at 9 years. In adjusted model, the odds (OR) of being anemic in 9 years of age was 1.81 (95% CI 1.07, 3.05; p =0.027) and 2.34 (1.37, 4.00; p =0.002) fold higher if their mothers were anemic at either GW14 or GW30, respectively, compared to non-anemic mothers **(Figure 6)**. The odds of having anemia at 9 years was even higher if the mothers were anemic at both time points (OR=3.05, 95% CI 1.42, 6.14; p =0.002). The odds increased, about 6 times (OR=5.92, 95% CI 2.64, 13.25; p <0.001) when they were also anemic at 4.5 years. However, no impact of maternal anemia was observed at 4.5 years of age (at GW14, OR=0.45, 95% CI 0.15, 1.34; p =0.15; at GW30, OR=1.28, 95% CI 0.54, 3.04; p = 0.56 and at both time points, OR=0.66, 95% CI 0.18, 2.43; p =0.54).

When the analyses were stratified by supplementation groups, the odds of having anemia at 9 years of age was 2.34 (95% CI 0.49, 11.04; p =0.28), 3.95 (95% CI 1.09, 14.38; p =0.03) and 4.15 (95% CI 1.25, 13.76; p =0.02) times higher in the Fe30F, Fe60F and MM groups, respectively, if their mothers were anemic both at GW14 and GW30 **(Figure 6)**. Again, the odds of having anemia at 9 years of age was 11.16 (95% CI 2.73, 45.59; p =0.001), 6.35 (95% CI 1.50, 26.81; p =0.01) and 3.50 (95% CI 0.76, 16.09; p =0.10) times higher in the Fe30F, Fe60F and MM groups, respectively, if they were anemic at early childhood (4.5 years).

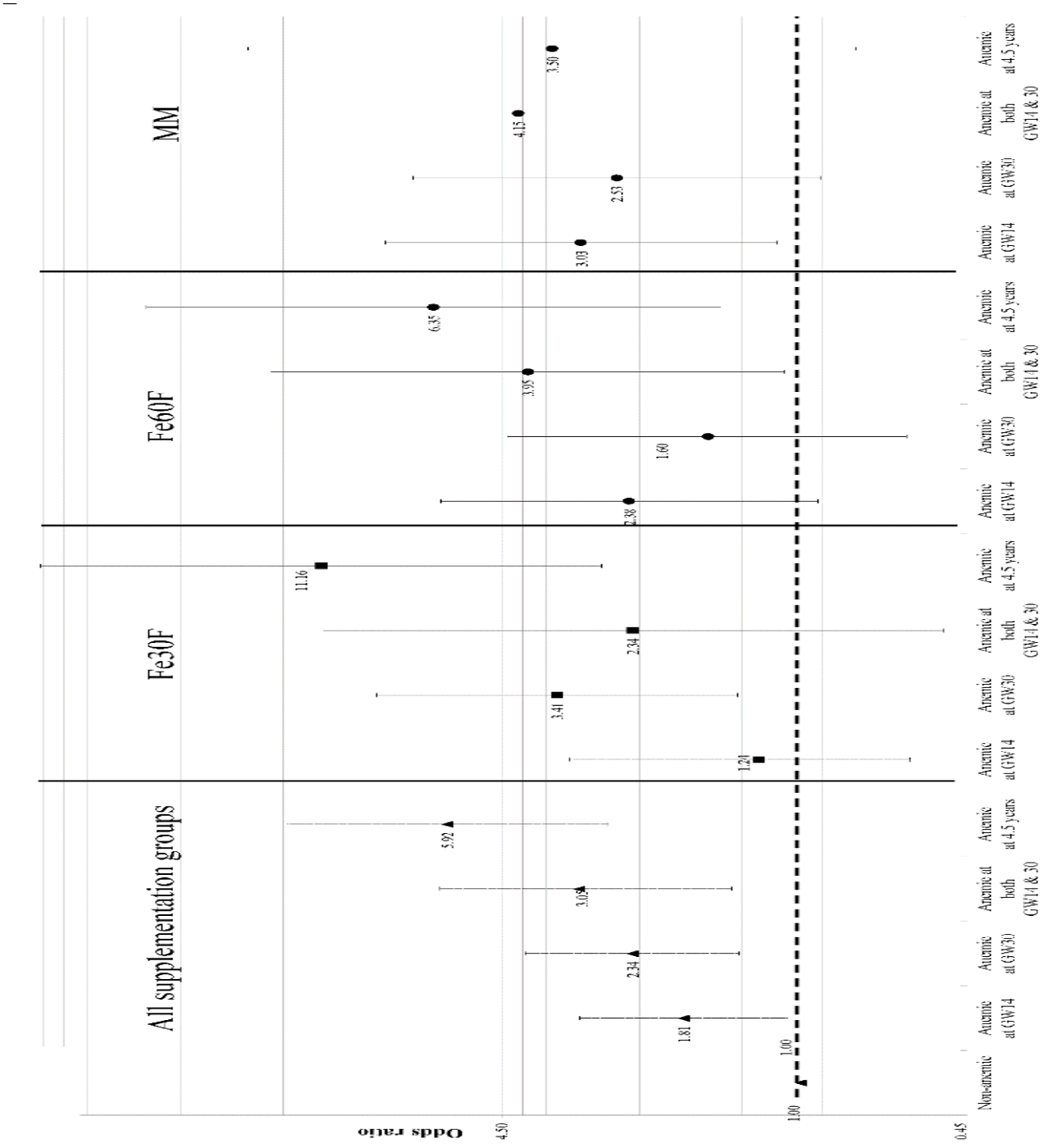

Figure 6: The odds of having anemia at 9 years of age if the mothers were anemic at GW14 or GW30 or if the children were anemic at 4.5 years of age.

3.7 Chronic arsenic exposure and telomere length (Paper II)

Adjusted regression models showed significant inverse associations between maternal (GW8) and childhood U-As (4.5 and 9 years of age) and TL at 9 years of age above the spline knot 5.5 (**Table 7**). In contrast, concurrent U-As below the spline knot was significantly positively associated with TL at 9 years of age. In 4.5 years old children, U-As at GW8 and concurrent U-As above the spline knot were inversely associated with TL, although the associations were not statistically significant. These associations remained unchanged when total available children (n=275) at 4.5 years of age were considered (**Figure 3**). The estimates of the associations between U-As at GW8 with TL at 4.5 (β=-9.7) and 9 years (β=-10.4) were similar (**Table 7**).

Table 7: Regression analysis of urinary arsenic concentration at gestation week 8, 4.5 and 9 years and arsenic metabolites at 9 years with telomere length at 4.5 and 9 years of age.

TL	Children at 4.5 years (n=213)	Children at 9 years (n=351)
	β (95% CI)[a]	β (95% CI)[a]
U-As at GW8		
<5.5[b]	7.1(-26.9, 41.0)	10.4(-8.4, 29.1)
>5.5	-9.7(-19.4, 0.05)[#]	-10.4(-16.9, -3.9)*
U-As at 4.5 years		
<5.5[b]	-8.7(-42.8, 25.4)	-3.3(-25.7, 19.2)
>5.5	-4.7(-16.0, 6.6)	-15.6(-23.6, -7.6)*
U-As at 9 years		
<5.5[b]	-	23.2(5.9, 41.6)*
>5.5	-	-33.9(-41.9, -25.9)*
MMA in urine (%)		
<7.0[b]	0.01(-0.08, 0.10)	0.02(-0.05, 0.08)
>7.0	-0.02(-0.05, 0.01)	-0.05(-0.07, -0.02)*
DMA in urine (%)		
<80.0[b]	0.01(-0.02, 0.03)	0.02(-0.004, 0.04)
>80.0	0.003(-0.02, 0.03)	0.02(-0.01, 0.04)
iAs in urine (%)		
<15.0[b]	0.02(-0.01, 0.04)	-0.001(-0.02, 0.02)
>15.0	-0.07(-0.13, -0.003)*	-0.03(-0.09, 0.02)

[a]Adjusted for child age, height-for-age z-score, gender, plasma C-reactive protein and total leukocytes at 9 years, family SES at 9 years, mothers' education.
[b]Spline regression model using spline knot at $\log_2$ urinary arsenic 5.5 (corresponding to 45µg/L), fraction of MMA at 7%, fraction of DMA at 80% and fraction of iAs at 15%.
*indicates $p<0.05$ and [#]indicates $p<0.10$

To evaluate long term changes of arsenic on TL, we evaluated the association of childhood exposure to arsenic in relation to TL at 4.5 and 9 years of age by mixed effect models. A significant inverse association was found between childhood arsenic exposure above the spline knot and childhood TL (β=-15.4, 95% CI= -22.19, -8.64; p<0.001) **(Figure 7)**. Adjustment with U-As at GW8 did not change the estimates.

The TL was significantly inversely associated with the fraction of MMA above the spline knot of 7% in 9 years old and above 15% for iAs in 4.5 years old children (**Table 7**) likely reflecting increased toxicity due to poor methylation of arsenic in these children.

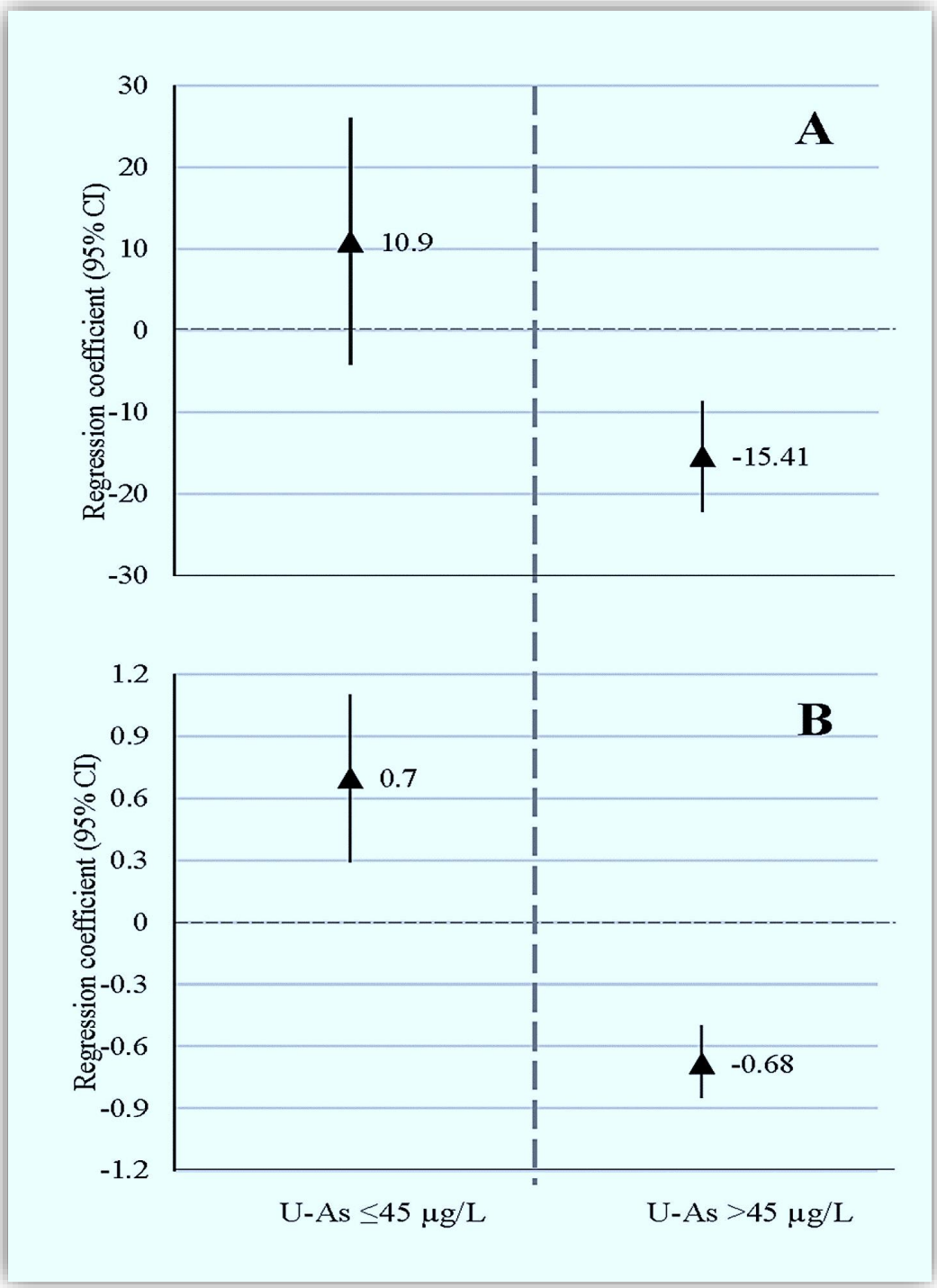

Figure 7: Linear mixed effects models of U-As over time (4.5 and 9 years of age) on outcome changes of telomere length (A) and sjTRECs (B) in children with a spline knot introduced at 5.5 (corresponding to 45 µg/L of U-As). The models were adjusted with covariates (child age, height-for-age z-score, gender, family SES at 4.5 and 9 years of age, plasma CRP concentration at 9 years, mother's education and U-As at GW 8.

3.8 Chronic arsenic exposure, sjTRECs and immune cells (Paper II)

Significant inverse associations were found between U-As at GW8, 4.5 and 9 years of age and sjTRECs at 9 years of age above the spline knot 5.5 (**Table 8**). Only in 9 years old children a significant positive association of sjTRECs was obtained with U-As below the spline knot. To examine whether elevated levels of sjTRECs were linked with illness in these children, we evaluated association between sjTREC concentrations and morbidity outcomes and found no association. However, children with U-As below the spline knot had higher counts of lymphocytes (p=0.01) and monocytes (p=0.05) but lower counts of total leukocyte (p=0.006), eosinophils (p=0.01) and platelets (p=0.01) compared to children with U-As above the spline knot. The presence of higher numbers of lymphocytes supported the finding of higher concentrations of sjTRECs in children with U-As below 5.5. No significant associations were noted between sjTREC concentrations at 4.5 years (considering either n=213 or n=275) and prenatal and concurrent arsenic exposures (**Figure 3; Table 8**).

The mixed effect model with spline function showed an inverse association between childhood arsenic exposure and sjTRECs (β=-0.68, 95% CI= -0.85, -0.49; p<0.001) above the spline knot at 5.5. However, a positive association was found between childhood arsenic exposure and sjTRECs in all children (β=0.70, 95% CI= 0.29, 1.10; p<0.001) below the spline knot **(Figure 7)**. Further the estimates remain unchanged after adjustment with U-As at GW8.

Significant negative correlation of MMA% with sjTRECs levels was found above spline knot of 7% at 9 years of age. No other significant association of sjTRECs was found with other arsenic metabolites at either time points (**Table 8**).

Table 8: Regression analysis of urinary arsenic concentration at gestation week 8, 4.5 and 9 years and arsenic metabolites at 9 years with sjTRECs at 4.5 and 9 years of age.

sjTRECs	4.5 years old children (n=213)	9 years old children (n=349)	
	Model 1 β (95% CI)[a]	Model 1 β (95% CI)[a]	Model 2 β (95% CI)[b]
U-As at GW8			
<5.5[c]	0.72 (-0.02, 1.5)	0.29(-0.23, 0.81)	0.32(-0.20, 0.85)
>5.5	-0.12 (-0.33, 0.09)	-0.43(-0.61, -0.25)*	-0.42(-0.61, -0.24)*
U-As at 4.5 years			
<5.5[c]	0.36 (-0.39, 1.1)	0.03(-0.58, 0.65)	0.09(-0.55, 0.73)
>5.5	-0.11 (-0.35, 0.14)	-0.62(-0.84, -0.40)*	-0.59(-0.82, -0.36)*
U-As at 9 years			
<5.5[c]	-	1.3(0.87, 1.8)*	1.3(0.80, 1.8)*
>5.5	-	-1.3(-1.5, -1.1)*	-1.3(-1.5, -1.1)*
MMA in urine (%)			
<7.0[c]	-0.03(-0.32, 0.26)	0.19(-0.04, 0.42)	0.04(-0.08, 0.16)
>7.0	-0.05(-0.14, 0.04)	-0.16(-0.24, -0.08)*	-0.26(-0.38, -0.14)*
DMA in urine (%)			
<80.0[c]	-0.03(-0.11, 0.06)	0.03(-0.04, 0.11)	0.05(-0.03, 0.14)
>80.0	0.04(-0.04, 0.13)	0.03(-0.04, 0.10)	0.02(-0.05, 0.10)
iAs in urine (%)			
<15.0[c]	-0.02(-0.10, 0.07)	0.01(-0.07, 0.08)	-0.01 (-0.09, 0.07)
>15.0	0.15(-0.06, 0.36)	0.04(-0.16, 0.23)	0.14 (-0.10, 0.38)

[a]Model 1. Adjusted for child age, height-for-age z-score, gender, plasma C-reactive protein and lymphocytes at 9 years, SES of the family at 9 years of age, mothers' education.
[b]Model 2. Variables additionally adjusted by 8-OHdG at 9 years.
[c]Spline regression model using spline knot at $\log_2$ urinary arsenic 5.5 (corresponding to 45µg/L), fraction of MMA at 7%, fraction of DMA at 80% and fraction of iAs at 15%.
*indicates $p<0.05$.

3.9 Arsenic exposure, oxidative stress, telomere length and sjTRECs (Paper II)

Prenatal and childhood urinary arsenic were significantly positively associated with plasma 8-OHdG at 9 years of age, but not at 4.5 years of age (**Table 9**). Again, linear regression analysis showed a significant inverse association between 8-OHdG and sjTRECs at 9 years of age (β=-0.17; 95% CI, -0.30, -0.04; *p*=0.01), but not at 4.5 years of age (β=-0.15; 95% CI, -0.37, 0.08; *p*=0.195). However, we did not observe similar associations between TL and 8-OHdG either at 4.5 or 9 years of age.

To investigate whether the estimated effects of arsenic on sjTRECs may be mediated via arsenic-induced oxidative stress, we adjusted the associations of prenatal and childhood U-As with sjTRECs by 8-OHdG in children at 9 years. The estimates of the associations of U-As with sjTRECs did not change after being additionally adjusted for 8-OHdG above or below the spline knot of 5.5 (**Table 8**). At 9 years, concentration of plasma 8-OHdG above the spline knot was significantly higher (mean 3.6 ng/mL) than below (mean 3.0 ng/mL) the spline knot (*p*=0.007), but this was not the case at 4.5 years of age.

Table 9: Regression analysis of urinary arsenic concentration at gestation week 8, 4.5 and 9 years with 8-OHdG at 4.5 and 9 years of age.

	8-OHdG at 4.5 years (n=213)		8-OHdG at 9 years (n=335)	
	Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]
U-As at GW8	-0.06(-0.20, 0.07)	-0.06(-0.19, 0.08)	0.13(0.02, 0.24)*	0.13(0.01, 0.24)*
U-As at 4.5 years	-0.07(-0.24, 0.09)	-0.08(-0.24, 0.09)	0.23(0.09, 0.36)*	0.22(0.08, 0.36)*
U-As at 9 years	-	-	0.30(0.17, 0.43)*	0.32(0.19, 0.45)*

Data were expressed as regression coefficient (β) and 95% confidence intervals
[a]Adjusted for child age, height-for-age z-score, gender, plasma C-reactive protein at 9 years, SES at 9 years of age, and mothers' education.
*indicates $p<0.05$.

CHAPTER 4
DISCUSSION

The studies that form the basis for this thesis were population based longitudinal studies on rural Bangladeshi women and their children with a background of wide use of arsenic contaminated tube-well water for drinking and cooking purposes. It is a community-based nutrition trial with a large pregnancy cohort (the MINIMat trial) that provided the prospect to determine even smaller increases in risk that might be of public health significance.

The analyses focus on epidemiological associations of prenatal and childhood (4.5 and 9 years) arsenic exposures with anemia related plasma biomarkers and immune markers in children. The present longitudinal studies have revealed that chronic arsenic exposure from *in utero* to pre-adolescent age do not influence the anemia status but has a profound adverse effect on immune function.

Data are conflicting in regards to the effect of arsenic on Hb. Binding of arsenic in a complex with glutathione in RBC (as seleno-*bis*-(S-glutathionyl) arsinium ion [$(GS)_2AsSe$]) is one mechanism of detoxification by the body (176). Naranmandura et al. (141) isolated and identified a major arsenic binding protein as the ternary DMA^{III}-Hb-haptoglobin complex in rat plasma after a single oral administration of arsenite. They suggested that DMA^{III}-Hb complex was first formed in RBCs of rats and then released on hemolysis into the plasma where it bound to haptoglobin to form the ternary complex. A study in West Bengal, India depicted that chronic arsenic exposure (mean±SD U-As, 391.49±226.67 µg/L in exposed versus 28.66±19.02 µg/L in unexposed) destructed human erythrocytes by destabilizing cell membranes resulting in release of Hb and thus increasing the risk of anemia (120). A higher prevalence of anemia particularly pregnancy anemia was reported among women with low-moderate level [median (25^{th}, 75^{th} %tile) U-As, 1.46 (0, 3.61) µg/L in anemic versus 0.10 (0, 2.39) µg/L in non-anemic] drinking water arsenic exposure (177).

In rural Bangladesh, a study in male adults revealed that even with arsenic exposure as high as [mean (range) U-As, 1730.4 (100–11160) μg/g], there was no effect on Hb but RBC counts were reduced. However, among a sub-group (smokers) an inverse association was found between U-As and Hb (124). Islam et al (178) demonstrated that chronic arsenic toxicity [median (range) U-As, 129.7 (20.0 – 1764.0) μg/L] was associated with poor nutritional status and anemia was present among 26% of adult patients with arsenicosis (i.e. skin lesions). However, they did not show any association between exposure and anemia. Similarly, in another study in Bangladesh using a larger sample size (n=1984), no association was found between arsenic exposure and Hb in adults with and without skin lesions (median water As, 39.0 μg/L). However, the influence of arsenic on Hb was only evident in male subjects (132). Further investigations in a sub-sample (n=147) from the above cohort among anemic and non-anemic women revealed that arsenic-induced skin lesions (median±interquartile range water As, 37.7±268.0 μg/L) is more common in anemic women (179). Merrill et al. (130) found no relationship between arsenic levels in drinking water (water As, >50 μg/L in 12% of tubewells) and prevalence of anemia among women of reproductive age. In contrast, increased maternal blood arsenic levels (mean±SD blood As, 12.4±3.4 μg/L in anemic versus 14.8±4.0 μg/L in non-anemic) were found to be related to decreased anemia during pregnancy in a longitudinal study (128).

There is limited data available in children. To study the association of anemia with heavy metals, a study in Mexico found that average arsenic level in dry blood was higher in the anemic children compared to non-anemic children (180). In a study in Mexican school children evaluating the associations of U-As (mean±SD, 58.1±33.2 μg/L) with several cognition tests found no association between U-As and Hb in these 6-7 years old children (181). The findings of the above

studies do not clearly show an association between arsenic and Hb; rather it indicates that low Hb levels serve to exacerbate the harmful health effects of chronic arsenic exposure.

Among the MINIMat children, even though there were about 22-40% undernourished children, the proportion of anemia was similar to normal children. We did not find any influence of chronic exposure on anemia in these children. It may be noted that the exposure levels of arsenic in most adult populations of the above studies were much higher than our MINIMat children. Moreover, the duration and magnitude of exposure to arsenic were lower in children (<9 years only) compared to pregnant mothers (median U-As: 88 μg/L in pregnancy, 57.1 μg/L in 4.5 years and 53.9 μg/L in 9 years). Furthermore, there was 40% reduction in the proportion of arsenic-contaminated tubewells in rural Bangladesh by the successful intervention of the Water, Sanitation and Hygiene (WASH) program of BRAC in collaboration with the government of Bangladesh (182) along with other mitigation programs. Installation of deep tubewells occurred in later years (after birth) in these locations could have contributed to relatively lower arsenic exposure in older children. This level of exposure might not be sufficient to influence the concentration of Hb and other anemia related biomarkers in the school-going children. The abundance of folate in the MINIMat children (without folate deficiency) may also have reduced arsenic toxicity via efficient metabolism since arsenic metabolic pathway is folate dependent (183). Moreover, our study-children were generally breastfed and thus were less exposed to arsenic during the critical window of development.

The prevalence of anemia in 9 year old MINIMat children (15%) was slightly lower than the prevalence rate of 19% obtained in the Bangladesh National Micronutrient Survey conducted in school-age (6-11 years) children in 2011-2012 (119). One recent study of school-based micronutrient fortification program in rural Bangladesh for children aged 6-11 years determined

slightly lower rate (12.5%) of anemia at baseline status (184). Earlier studies have shown much higher rates of anemia in children. According to the WHO database on anemia in Bangladesh, the prevalence of anemia in school-age children (5-12 years) was 34% in 2007 (185). The anemia prevalence survey carried out by Bangladesh Bureau of Statistics/UNICEF in 2003 demonstrated about 46% anemia in rural Chittagong Hill Tracts and 24% anemia in urban Dhaka in 13-14 year old boys and girls (186). A nutritional survey conducted in 2001 by Helen Keller International (HKI) in collaboration with the Institute of Public Health Nutrition showed that 33.5% of the school-age children (5-11 years) in rural Bangladesh suffered from anemia (187). The reasons for decline in anemia prevalence in this age group could be multi-factorial. (i) One factor could be the high iron content in groundwater in the rural areas where the main source of drinking and cooking water is from tubewells. The Bangladeshi standard for iron in drinking water is 0.3-1.0 mg/L (188). British Geological Survey demonstrated that 23% of tubewell water contained >5 mg/L where 10% had >10 mg/L iron in Bangladesh (189). In Matlab, the Fe concentrations in 78% of the shallow wells (<50 meters) were above the recommended limit of 0.3 mg/L of WHO and United States Environmental Protection Agency (190). Again in 2014, Bhattacharya et al showed that the mean iron content in 243 tubewells in Matlab was 4.2 mg/L which was 14 fold higher than the WHO recommended cut-off for iron in drinking water (191). Merrill et al. demonstrated that groundwater contributed significantly to the daily iron intake of rural women which might protect them from ID and IDA; the iron intake through water and food was compared with iron status in the body (192). According to the National Micronutrient Survey, low prevalence of iron deficiency in preschool and school-age children, and non- pregnant, and non-lactating women was associated with frequent use of tubewell water and low IDA was attributed to consumption of high iron containing groundwater (119). In agreement with these findings, 99.3% of the children in the

present study had normal ferritin levels indicating lack of iron deficiency. (ii) Another factor could be the high coverage of vaccination (84%) and vitamin A supplementation (93%) by the age of 23-59 months (according to survey of Expanded Programme on Immunization 2014), various nutritional intervention programs for mothers and children (such as Bangladesh Maternal Infant and Young Child Nutrition-Home Fortification Programme; The National Food Security Nutritional Surveillance Project; The Nutritional Surveillance Project of HKI; the Poverty Reduction Strategy and the Health, Nutrition and Population Sector Program for 2001-13)- all of which may have contributed to improved overall health and nutritional status, resulting in reduced mortality and morbidity due to infectious diseases (193). According to Bangladesh Demographic and Health Survey, the level of stunting has declined from 51% to 36% and underweight from 43% to 33% among children below 5 years of age in the last decade (from 2004 to 2014) (194). In a study carried out in Matlab during 2000-2012, Das et al. also demonstrated a marked reduction in stunting and underweight among under-five children (195). (iii) Prevalence of diarrhea in Bangladesh has declined from 7% in 2007 to 4.3% in 2014 (194). Such health indicators suggest a positive impact on the health outcomes in children of rural Bangladesh all of which may contribute to reduced anemia.

We have found that none of the children had folate deficiency; iron (8% or 0.2%) and vitamin B_{12} deficiencies (0.9%) were also minimal suggesting that a major cause of anemia in children was not related to iron, folate or B_{12} deficiency. These results are in line with the findings at 4.5 years as reported earlier on MINIMat children (196). The presence of normocytic normochromic anemia in more than half (about 61%) of the anemic children could indicate presence of recent infections, deficiency of vitamin B_2 (riboflavin) and genetic disorders such as sickle cell anemia, red blood cell membrane disorders etc. that may cause hemoglobinopathies in

children of this age group (197). Microcytic anemia can be attributed to IDA, vitamin B_6 (pyridoxine) deficiency and thalassemia (197). The exact nature of anemia (whether genetic or B_2 or B_6 deficiency) could not be determined in these children, however, parents were notified, and suspected children were referred to specialists.

When we investigated the consequence of long-term effects of maternal nutrition supplementation in the 9 years old children, we have found that Hb concentrations did not differ by supplementation groups in children. This finding was in line with the original MINIMat trial where supplementation did not affect Hb concentration in pregnant women at GW30 (154). In the majority of the children (85-95%), Hb concentration was well within the normal range as earlier reported in a larger MINIMat cohort (n=1354) (196). The positive association between Hb and plasma concentration of ferritin, B_{12}, hepcidin and vitamin A mainly in Fe60F group (but not in the Fe30F and MM groups) indicated a beneficial impact of the higher dose of antenatal iron on Hb concentration in 9 years old children. Vitamin A has an important role in iron mobilization into Hb of developing RBC (117). Iron status impacts plasma and liver levels of vitamin A (198). Supplementation with 60 mg iron and folate during pregnancy increased hepcidin concentration in iron deficient Tanzanian women (serum ferritin $\leq$12 μg/L) (199). In support of our study, iron deficiency was low among pregnant women in rural Bangladesh who showed positive association between Hb levels and plasma concentrations of Zn, vitamin B_{12}, and α-tocopherol (200). Improved outcome of iron supplementation on Hb recovery was observed in anemic or iron deficient children but not in adolescents or adults (201).

We found beneficial effects of multiple micronutrients (MM) on child nutritional status (BAZ scores). In a larger cohort of MINIMat children (n=1634), Khan et al showed that maternal MM supplementation increased the proportion of stunting in boys only at 4.5 years of age (202).

However, we did not find any impact of MM supplementation on the proportion of stunting or underweight children either at 4.5 or 9 years of age, not even in boys. Data on child growth beyond 5 years of age in relation to maternal supplementation are scarce. A meta-analysis of randomized controlled trials reported that maternal multi-micronutrient supplementation had a significant positive effect on head circumference of under-5 children without any effects on weight, height, WAZ, HAZ and WHZ (203). In a randomized controlled trial in Vietnam, children born to mothers who received low-dose iron (120 mg per week) during pregnancy had significantly lower height and HAZ compared to children born to mothers who received higher-dose (420 mg per week) antenatal iron supplementation regimens (204).

In our study, supplementation with MM also markedly reduced markers of inflammation and infection in 9 year old children. The MM supplement of UNICEF/WHO/UNU contained important antioxidants such as vitamin E, vitamin C and selenium which are known to reduce inflammatory responses. It is possible that prenatal MM supplementation improved the immune reserve of the growing fetus that persists and aid in combating infections in later childhood. Very few studies have reported effects of MM supplementation during pregnancy on disease outcomes of older children. Multi-vitamin supplementation of HIV-infected women during pregnancy and lactation was shown associated with reduced rate of all types of diarrhea among under-5 HIV-negative children (205). Maternal vitamin A supplementation enhanced natural antibody concentrations in Nepalese children at preadolescent age (9 to 13 years); the authors postulated that pre-natal supplementation leads to a higher reservoir and sustained natural immunity in these children (206). An increasing body of evidence suggests that maternal nutritional status including micronutrients, life-style, exposure to pollutants etc. from preconception through lactation causes

fetal or neonatal epigenetic changes that might account for altered mechanisms of growth, metabolism and diseases observed later in life (207).

One important finding of this study was that anemia during pregnancy and in early childhood was an important risk factors for anemia in pre-adolescent age **(Paper I)**. Several studies indicate that the nutritional background of a woman during pregnancy and especially during early life is a critical determinant of her offspring's subsequent health outcomes such as suboptimum growth and mortality, suggesting an intergenerational transfer of poor health from mother to child (208, 209). Maternal conditions such as anemia affecting the gestational environment can cause fetal or neonatal epigenetic changes that accounts for altered mechanisms of growth and metabolism observed later in childhood (207). Indeed, a study in Burma reported association between maternal anemia and anemia in 6-36 months old children (210). To the best of our knowledge, this is the first report that shows that this risk is also apparent at 9 years of age. A study involving 3 generations of Sami people in Sweden exhibited the age of 8-12 years as a sensitive period where food availability might induce epigenetic or other changes that could be transmitted to the next generations impacting health outcomes (211). In line with this, we also found that the influence of maternal anemia during pregnancy emerged in pre-adolescent age (9 years) and not earlier at 4.5 years. An interesting finding of the present study was that Hb level at 4.5 years of age was a stronger risk factor than maternal Hb (~2 folds higher) for being anemic in 9 years of age. The findings reflect that early childhood nutritional status is also very important besides prenatal nutrition for optimum health outcome at later age and highlighting the public health importance of these findings.

We studied the effects of arsenic on immune function in 9 years old children. Our findings exhibited that persistent exposure to arsenic from fetal life up to 9 years reduced telomere length

and thymic output in PBMC where concurrent exposure had the strongest effect **(Paper II)**. There is mounting interest in studies on environmental exposures and telomere attrition which have mostly been carried out in adults in cross-sectional studies. However, longitudinal birth cohort studies are lacking. We found that arsenic concentrations that are considered safe in Bangladesh (cut-off <50 μg/L) are actually affecting the immune system at relatively low levels. Alteration in the immune system are already apparent at relatively low levels of arsenic (27% of the wells in Matlab exceeded the national standard (33)). Here we found that prenatal and persistent childhood exposure to arsenic above 45 μg/L of U-As reduced TL in children. For every doubling of arsenic exposure, there was a decrease in 34 kb/dg of telomere length in 9 years old children. One study showed that adults with chronic exposure to arsenic (average total U-As ≥19.3 μg/L) have shortened TL in leukocytes in presence of hOGG1 Cys polymorphism indicating that arsenic-mediated telomere shortening was influenced by defects in DNA excision repair (212). However, other epidemiological studies in adults have shown longer TL in PBMC in relation to high arsenic exposure. One study in West Bengal (213) reported that people with arsenic-induced skin lesions (mean U-As 290 μg/L) exhibited telomerase-independent elongation of TL compared to subjects with lower exposure (mean U-As 30.5 μg/L). Studies conducted in people chronically exposed to high arsenic showed significantly longer telomeres being associated with higher urinary arsenic (median U-As 230 μg/L (96); 80-196 μg/L (94); mean U-As 856.0 μg/g of creatinine (95)). In the latter three studies, poor arsenic methylation efficiency and elongated TL appeared to play an important role in arsenic-related carcinogenesis. In our 9 years old children, higher fraction of MMA (above the spline knot of 7%) also decreased TL significantly. Therefore, poor methylation efficiency was found to be related with shortening of TL among these children. The seemingly counterintuitive findings of shortening or elongation of TL by arsenic exposure could depend on

various environmental and other factors (such as arsenic dose, duration of exposure, age, DNA repair mechanisms) that can manipulate the telomere length maintenance machinery and have opposing directions of effects (214, 215). For example, median U-As concentrations in our study (88, 57 and 54 µg/L at GW8, 4.5 years and 9 years respectively) were much lower than the above studies. Ours was a birth cohort study and the negative association of arsenic exposure (above U-As of 45 µg/L) with TL remained evident at each time point as opposed to the above cross-sectional, case-control studies in adults. It is plausible that far longer duration of arsenic exposure in adults compared to children influences the outcome.

In contrast to the above findings, we found that U-As concentrations below 45 µg/L appeared to be associated with longer telomeres in the children. An *ex vivo* study demonstrated that treatment with lower concentration (0.0001 µM) of iAs markedly increased TL in cord blood leukocytes but at higher concentration (1 µM) significantly decreased the TL; this occurred in parallel to decreased telomerase expression (82). Similarly, an *in vitro* study (216) indicated that arsenite at low concentrations (<1 µM) promoted telomerase activity and maintained TL in cell lines, while at high concentrations (>1 µM) there was drastic reduction in TL and increased apoptosis. Treatment with arsenic (0.75 µM) inhibited telomerase transcription and resulted in TL shortening and chromosomal end lesions with a dominance of chromosomal end-to-end fusions in yet another *in vitro* study (217). Thus, shorter telomeres may promote genomic instability and initiation of carcinogenesis or reduce cell survival through enhanced apoptosis (218). There may be a critical threshold of arsenic exposure beyond which exposed cells either undergo attrition or elongation of TL. However, it is difficult to directly compare the *in vitro* conditions with far more complex *in vivo* microenvironment.

We have earlier shown in the MINIMat cohort that arsenic exposure reduced thymic size (3) and decreased thymic output in infants/neonates reflected by reduced sjTRECs levels (6). Here we extend those findings by demonstrating that both prenatal and childhood arsenic exposure decreased sjTRECs levels at 9 years of age with progressively stronger association being evident with concurrent exposure. Thymic involution begins from an early age of 1 year and with progression of age a shift occurs from efficient thymic lymphopoiesis to T-cell generation through peripheral replication which becomes the dominant mechanism of replenishing the T-cell pool (219). Decrease in sjTREC concentrations reflects immunodeficiency, a well-known phenomenon in clinical conditions including HIV-infection (220, 221), chemotherapy, bone marrow transplantation, severe respiratory syncytial virus (RSV) infections in neonates (222). Several trials of HAART (highly active antiretroviral therapy) treatment of HIV/AIDS patients, both adult and pediatric, have demonstrated regeneration of sjTRECs containing T lymphocytes and recovery from immunodeficiency (223, 224). Thus, reduction of sjTRECs due to arsenic exposure suggests depletion of T cell pool in the children eventually leading to immunodeficiency. Our findings are in keeping with previous studies demonstrating reduced frequency of T cells in children and adults with chronic arsenic exposure (76, 77, 79). One of the mechanisms of immunosuppressive effects of arsenic is to induce cell apoptosis (77). We have previously shown that arsenic exposure during pregnancy reduced T cell counts in the placenta and cord blood and upregulated apoptosis related genes in cord blood (6, 8). Childhood arsenic exposure reduced T cell-mediated function in these MINIMat children at 4.5 years (9) and impaired mumps-vaccine specific responses at 9 years of age (10). T cells are required for an effective adaptive immune response. Depletion of T cells is likely to hamper adaptive immunity while aging T lymphocytes (with shorter TL) are hyporesponsive to infection or vaccination (225). Thus, our findings of inadequate production of

sjTRECs or naïve T cells and cellular exhaustion due to persistent arsenic exposure in childhood are closely linked to accelerated aging of immune cells (immunosenescence) that may subsequently result in mounting of suboptimal immune responses and increased disease susceptibility. Recent reports suggest that having a short or long TL may be largely established early in life and serve as a marker of susceptibility to chronic diseases and cancer in later life (226, 227).

In vitro and experimental studies have revealed that oxidative stress causes telomere attrition (228). Our earlier studies have shown that arsenic exposure during pregnancy increased 8-OHdG expression with increased inflammatory responses and reduced T cells in the placenta and cord blood (6, 8). In support of our early reports, we found that prenatal and childhood arsenic exposure increased oxidative stress, and again oxidative stress appeared to reduce sjTRECs levels in 9 years old children but not at an earlier age of 4.5 years. However, as plasma 8-OHdG did not affect the relationship between U-As and sjTRECs, the mechanisms of 8-OHdG-mediated oxidative damage of naïve T cells may be distinct from arsenic-induced oxidative damage with little overlap. The possible modes of action of arsenic toxicity other than increased oxidative stress are direct genotoxic effects, altered expression of growth factors, impairment of enzymes involved in DNA synthesis and repair (229).

Follow-up studies in MINIMat children who are exposed from *in utero* will give a unique opportunity to help gain better insights into the mechanisms by which health in later life is influenced.

5 CONCLUSIONS

Our results indicate that maternal micronutrient supplementation confers beneficial health effects on pre-adolescent school-age children although the effects were not apparent at an earlier age of 4.5 years. The prevalence of anemia in 9 years old children in Matlab, Bangladesh was lower than expected; however, it remains a public health problem and debates are ongoing whether it is still a necessity to continue programs concerning anemia alleviation in Bangladesh. Anemia during pregnancy and in early childhood appeared to be important risk factors for anemia in pre-adolescent age. This information might aid the public health services to take initiatives aimed at reducing anemia and micronutrient deficiencies in early childhood in addition to the programs being carried out for women during pregnancy.

Our findings also suggest that chronic arsenic exposure from early life, even at relatively low exposure, can result in TL attrition and lower production of naïve T cells potentially leading to immunosenescence and immunodeficiency. Therefore, environmental exposure to arsenic during early life may result in lifelong changes in health trajectories. In summary, we may draw the following conclusions from this thesis:

- Arsenic exposure had no effect on Hb levels and anemia related biomarkers (sTfR, ferritin, vitamin B_{12}, folate, vitamin A, hepcidin, zinc) in 9 years old school-going children.
- About 28% of the women were found to be anemic at GW14, 35% at GW30 and 23% at both time points.
- The prevalence of anemia was 5% in 4.5 and 15% in 9 years old children.

- Anemia during early childhood was a greater risk factor than pregnant mother's anemia (during pregnancy) for these children. The risk of anemia in 9 years old children
    - was 3-fold higher if their mothers were anemic during pregnancy.
    - increased (about 6 times) when children were also anemic at a younger age (4.5 years).
- 2.5% children had IDA according to sTfR cut-off and 0.2% (one child) when applying the ferritin cut-off.
- Multiple micronutrient supplementation during pregnancy had positive effects on nutritional status (improved BMI z-scores) and inflammation markers (CRP) in 9 years old children.
- Persistent arsenic exposure from fetal life up to 9 years was associated with reduced

    -Telomere Length in PBMC

    -Naïve T cells and thymic output (sjTRECs)

    with strongest effects being observed at concurrent exposure
- Significant negative correlation of MMA% with TL and sjTRECs levels were found above spline knot of 7% in 9 years old children.
- With prenatal and childhood arsenic exposure, increased oxidative stress (8-OHdG) was evident at 9 years of age, but not at an earlier age of 4.5 years.

6 REFERENCES

1. Farzan SF, Li Z, Korrick SA, Spiegelman D, Enelow R, Nadeau K, et al. Infant Infections and Respiratory Symptoms in Relation to in Utero Arsenic Exposure in a U.S. Cohort. Environ Health Perspect. 2016;124(6):840-7.
2. Rahman A, Vahter M, Ekstrom EC, Persson LA. Arsenic exposure in pregnancy increases the risk of lower respiratory tract infection and diarrhea during infancy in Bangladesh. Environ Health Perspect. 2011;119(5):719-24.
3. Raqib R, Ahmed S, Sultana R, Wagatsuma Y, Mondal D, Hoque AM, et al. Effects of in utero arsenic exposure on child immunity and morbidity in rural Bangladesh. Toxicol Lett. 2009;185(3):197-202.
4. Smith AH, Yunus M, Khan AF, Ercumen A, Yuan Y, Smith MH, et al. Chronic respiratory symptoms in children following in utero and early life exposure to arsenic in drinking water in Bangladesh. Int J Epidemiol. 2013;42(4):1077-86.
5. Dangleben NL, Skibola CF, Smith MT. Arsenic immunotoxicity: a review. Environ Health. 2013;12(1):73.
6. Ahmed S, Ahsan KB, Kippler M, Mily A, Wagatsuma Y, Hoque AM, et al. In utero arsenic exposure is associated with impaired thymic function in newborns possibly via oxidative stress and apoptosis. Toxicol Sci. 2012;129(2):305-14.
7. Ahmed S, Akhtar E, Roy A, von Ehrenstein OS, Vahter M, Wagatsuma Y, et al. Arsenic exposure alters lung function and airway inflammation in children: A cohort study in rural Bangladesh. Environ Int. 2017;101:108-16.
8. Ahmed S, Mahabbat-e Khoda S, Rekha RS, Gardner RM, Ameer SS, Moore S, et al. Arsenic-associated oxidative stress, inflammation, and immune disruption in human placenta and cord blood. Environ Health Perspect. 2011;119(2):258-64.
9. Ahmed S, Moore SE, Kippler M, Gardner R, Hawlader MD, Wagatsuma Y, et al. Arsenic exposure and cell-mediated immunity in pre-school children in rural Bangladesh. Toxicol Sci. 2014;141(1):166-75.
10. Raqib R, Ahmed S, Ahsan KB, Kippler M, Akhtar E, Roy AK, et al. Humoral Immunity in Arsenic-Exposed Children in Rural Bangladesh: Total Immunoglobulins and Vaccine-Specific Antibodies. Environ Health Perspect. 2017;125(6):067006.
11. IARC. Some drinking-water disinfectants and contaminants, including arsenic. IARC Monogr Eval Carcinog Risks Hum. 2004;84:1-477.
12. WHO. Iron deficiency anaemia: assessment, prevention, and control. A guide for programme managers. Geneva; 2001.
13. Bhattacharya P, Jacks G, Ahmed KM, Routh J, Khan AA. Arsenic in groundwater of the Bengal delta plain aquifers in Bangladesh. Bull Environ Contam Toxicol. 2002;69(4):538-45.
14. Cullen WR, Reimer KJ. Arsenic speciation in the environment. Chem Rev. 1989;89(4):713-64.
15. Goldfrank L, Flomenbaum, N. E., Lewin, N. E., Howland, M. A., Hoffman, R. S., Nelson, L. S. Goldfrank's toxicologic emergencies. 7th ed. New York: McGraw-Hill Medical Publishing Division,; 2002.
16. Mandal BK, Suzuki KT. Arsenic round the world: a review. Talanta. 2002;58(1):201-35.

17. IARC. Working Group on the Evaluation of Carcinogenic Risks to Humans. Cobalt in hard metals and cobalt sulfate, gallium arsenide, indium phosphide and vanadium pentoxide. IARC Monogr Eval Carcinog Risks Hum. 2006;86:1-294.
18. IARC. Working Group on the Evaluation of Carcinogenic Risks to Humans. Arsenic, metals, fibres, and dusts. IARC Monogr Eval Carcinog Risks Hum. 2012;100(Pt C):11-465.
19. ATSDR. (Agency for Toxic Substances and Disease Registry). Toxicological profile for Arsenic. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service; 2007.
20. Amini M, Abbaspour KC, Berg M, Winkel L, Hug SJ, Hoehn E, et al. Statistical modeling of global geogenic arsenic contamination in groundwater. Environ Sci Technol. 2008;42(10):3669-75.
21. Naujokas MF, Anderson B, Ahsan H, Aposhian HV, Graziano JH, Thompson C, et al. The broad scope of health effects from chronic arsenic exposure: update on a worldwide public health problem. Environ Health Perspect. 2013;121(3):295-302.
22. Guo H, Stuben D, Berner Z. Adsorption of arsenic(III) and arsenic(V) from groundwater using natural siderite as the adsorbent. J Colloid Interface Sci. 2007;315(1):47-53.
23. Kamsonlian S SS, Majumder CB and Chand S. Biosorption of arsenic from contaminated water onto solid Psidium guajava leaf surface: equilibrium, kinetics, thermodynamics, and desorption study. Bioremediation Journal. 2012a;16(2):97-112.
24. Pennesi C VF, Totti C, Romagnoli T and Beolchini F. Nonliving biomass of marine macrophytes as arsenic(V) biosorbents. Journal of Applied Phycology. 2012;24(6):1495-502.
25. Bundschuh J, Nath B, Bhattacharya P, Liu CW, Armienta MA, Moreno Lopez MV, et al. Arsenic in the human food chain: the Latin American perspective. Sci Total Environ. 2012;429:92-106.
26. NRC. Arsenic in Drinking Water. Update. Washington, D C. National Academy Press.; 2001.
27. Wang JP, Maddalena R, Zheng B, Zai C, Liu F, Ng JC. Arsenicosis status and urinary malondialdehyde (MDA) in people exposed to arsenic contaminated-coal in China. Environ Int. 2009;35(3):502-6.
28. Schmidt CW. Arsenical association: inorganic arsenic may accumulate in the meat of treated chickens. Environ Health Perspect. 2013;121(7):A226.
29. Schmidt CW. In search of "just right": the challenge of regulating arsenic in rice. Environ Health Perspect. 2015;123(1):A16-9.
30. Steer L, Evans A. Bangladesh's progress in health: healthy partnerships and effective pro-poor targeting. Overseas Development Institute, London UK:Development Progress, ODI Publications; 2011.
31. UNICEF, Bangladesh Bureau of Statistics (BBS). Multiple Indicator Cluster Survey (MICS). Bangladesh National Drinking Water Quality Survey Of 2009. 2011.
32. Datta BK, Bhar MK, Patra PH, Majumdar D, Dey RR, Sarkar S, et al. Effect of environmental exposure of arsenic on cattle and poultry in nadia district, west bengal, India. Toxicol Int. 2012;19(1):59-62.
33. Kippler M, Skroder H, Rahman SM, Tofail F, Vahter M. Elevated childhood exposure to arsenic despite reduced drinking water concentrations--A longitudinal cohort study in rural Bangladesh. Environ Int. 2016;86:119-25.

34. Edmunds WM, Ahmed KM, Whitehead PG. A review of arsenic and its impacts in groundwater of the Ganges-Brahmaputra-Meghna delta, Bangladesh. Environ Sci Process Impacts. 2015;17(6):1032-46.
35. Hossain M, Bhattacharya P, Frape SK, Jacks G, Islam MM, Rahman MM, et al. Sediment color tool for targeting arsenic-safe aquifers for the installation of shallow drinking water tubewells. Sci Total Environ. 2014;493:615-25.
36. Ravenscroft P, Brammer H, Richards K. Arsenic Pollution: A Global Synthesis.2009.
37. Vahter M. Mechanisms of arsenic biotransformation. Toxicology. 2002;181-182:211-7.
38. Marafante E, Vahter M, Norin H, Envall J, Sandstrom M, Christakopoulos A, et al. Biotransformation of dimethylarsinic acid in mouse, hamster and man. J Appl Toxicol. 1987;7(2):111-7.
39. Vahter M, Norin H. Metabolism of 74As-labeled trivalent and pentavalent inorganic arsenic in mice. Environ Res. 1980;21(2):446-57.
40. Vahter M, Envall J. In vivo reduction of arsenate in mice and rabbits. Environ Res. 1983;32(1):14-24.
41. Hayakawa T, Kobayashi Y, Cui X, Hirano S. A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase Cyt19. Arch Toxicol. 2005;79(4):183-91.
42. Rehman K, Naranmandura H. Arsenic metabolism and thioarsenicals. Metallomics. 2012;4(9):881-92.
43. Vahter M, Concha G. Role of metabolism in arsenic toxicity. Pharmacol Toxicol. 2001;89(1):1-5.
44. Vahter M. Effects of arsenic on maternal and fetal health. Annu Rev Nutr. 2009;29:381-99.
45. Skroder Loveborn H, Kippler M, Lu Y, Ahmed S, Kuehnelt D, Raqib R, et al. Arsenic Metabolism in Children Differs From That in Adults. Toxicol Sci. 2016;152(1):29-39.
46. Sun G, Xu Y, Li X, Jin Y, Li B, Sun X. Urinary arsenic metabolites in children and adults exposed to arsenic in drinking water in Inner Mongolia, China. Environ Health Perspect. 2007;115(4):648-52.
47. Tseng CH. A review on environmental factors regulating arsenic methylation in humans. Toxicol Appl Pharmacol. 2009;235(3):338-50.
48. Mass MJ, Tennant A, Roop BC, Cullen WR, Styblo M, Thomas DJ, et al. Methylated trivalent arsenic species are genotoxic. Chem Res Toxicol. 2001;14(4):355-61.
49. Petrick JS, Ayala-Fierro F, Cullen WR, Carter DE, Vasken Aposhian H. Monomethylarsonous acid (MMA(III)) is more toxic than arsenite in Chang human hepatocytes. Toxicol Appl Pharmacol. 2000;163(2):203-7.
50. Styblo M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, et al. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. Arch Toxicol. 2000;74(6):289-99.
51. Tuutijarvi T. Arsenate removal from water by adsorption with magnetic nanoparticles (γ – Fe2O3). Espoo: Aalto University, Finland; 2013.
52. WHO. Worldwide prevalence of anemia 1993-2005:WHO global database on anemia. Geneva; 2008.
53. Evens AM, Tallman MS, Gartenhaus RB. The potential of arsenic trioxide in the treatment of malignant disease: past, present, and future. Leuk Res. 2004;28(9):891-900.
54. Tapio S, Grosche B. Arsenic in the aetiology of cancer. Mutat Res. 2006;612(3):215-46.

55. Vahidnia A, van der Voet GB, de Wolff FA. Arsenic neurotoxicity--a review. Hum Exp Toxicol. 2007;26(10):823-32.
56. Farzan SF, Karagas MR, Chen Y. In utero and early life arsenic exposure in relation to long-term health and disease. Toxicol Appl Pharmacol. 2013;272(2):384-90.
57. Rahman A, Persson LA, Nermell B, El Arifeen S, Ekstrom EC, Smith AH, et al. Arsenic exposure and risk of spontaneous abortion, stillbirth, and infant mortality. Epidemiology. 2010;21(6):797-804.
58. Rahman A, Vahter M, Ekstrom EC, Rahman M, Golam Mustafa AH, Wahed MA, et al. Association of arsenic exposure during pregnancy with fetal loss and infant death: a cohort study in Bangladesh. Am J Epidemiol. 2007;165(12):1389-96.
59. Rahman A, Vahter M, Smith AH, Nermell B, Yunus M, El Arifeen S, et al. Arsenic exposure during pregnancy and size at birth: a prospective cohort study in Bangladesh. Am J Epidemiol. 2009;169(3):304-12.
60. Vahter M. Health effects of early life exposure to arsenic. Basic Clin Pharmacol Toxicol. 2008;102(2):204-11.
61. Hamadani JD, Tofail F, Nermell B, Gardner R, Shiraji S, Bottai M, et al. Critical windows of exposure for arsenic-associated impairment of cognitive function in pre-school girls and boys: a population-based cohort study. Int J Epidemiol. 2011;40(6):1593-604.
62. Flora SJ. Arsenic-induced oxidative stress and its reversibility. Free Radic Biol Med. 2011;51(2):257-81.
63. Kitchin KT, Wallace K. The role of protein binding of trivalent arsenicals in arsenic carcinogenesis and toxicity. J Inorg Biochem. 2008;102(3):532-9.
64. Hansen JM, Zhang H, Jones DP. Differential oxidation of thioredoxin-1, thioredoxin-2, and glutathione by metal ions. Free Radic Biol Med. 2006;40(1):138-45.
65. Mizumura A, Watanabe T, Kobayashi Y, Hirano S. Identification of arsenite-and arsenic diglutathione-binding proteins in human hepatocarcinoma cells. Toxicol Appl Pharmacol. 2010;242(2):119-25.
66. Walter I, Schwerdtle T, Thuy C, Parsons JL, Dianov GL, Hartwig A. Impact of arsenite and its methylated metabolites on PARP-1 activity, PARP-1 gene expression and poly(ADP-ribosyl)ation in cultured human cells. DNA Repair (Amst). 2007;6(1):61-70.
67. Ganyc D, Talbot S, Konate F, Jackson S, Schanen B, Cullen W, et al. Impact of trivalent arsenicals on selenoprotein synthesis. Environ Health Perspect. 2007;115(3):346-53.
68. Cohen SM, Arnold LL, Beck BD, Lewis AS, Eldan M. Evaluation of the carcinogenicity of inorganic arsenic. Crit Rev Toxicol. 2013;43(9):711-52.
69. Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L. An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. Environ Health Perspect. 2011;119(1):11-9.
70. Ye P, Kirschner DE. Reevaluation of T cell receptor excision circles as a measure of human recent thymic emigrants. J Immunol. 2002;168(10):4968-79.
71. Solana R, Pawelec G. Immunosenescence. NeuroImmune Biology. 2004;4:9-21.
72. Maicher A, Kastner L, Dees M, Luke B. Deregulated telomere transcription causes replication-dependent telomere shortening and promotes cellular senescence. Nucleic Acids Res. 2012;40(14):6649-59.
73. Descotes J. Importance of immunotoxicity in safety assessment: a medical toxicologist's perspective. Toxicol Lett. 2004;149(1-3):103-8.

74. Vial T, Choquet-Kastylevsky G, Descotes J. Adverse effects of immunotherapeutics involving the immune system. Toxicology. 2002;174(1):3-11.
75. Vial T, Descotes J. Immune-mediated side-effects of cytokines in humans. Toxicology. 1995;105(1):31-57.
76. Hernandez-Castro B, Doniz-Padilla LM, Salgado-Bustamante M, Rocha D, Ortiz-Perez MD, Jimenez-Capdeville ME, et al. Effect of arsenic on regulatory T cells. J Clin Immunol. 2009;29(4):461-9.
77. Rocha-Amador DO, Calderon J, Carrizales L, Costilla-Salazar R, Perez-Maldonado IN. Apoptosis of peripheral blood mononuclear cells in children exposed to arsenic and fluoride. Environ Toxicol Pharmacol. 2011;32(3):399-405.
78. Smith AH, Marshall G, Yuan Y, Liaw J, Ferreccio C, Steinmaus C. Evidence from Chile that arsenic in drinking water may increase mortality from pulmonary tuberculosis. Am J Epidemiol. 2011;173(4):414-20.
79. Soto-Pena GA, Luna AL, Acosta-Saavedra L, Conde P, Lopez-Carrillo L, Cebrian ME, et al. Assessment of lymphocyte subpopulations and cytokine secretion in children exposed to arsenic. FASEB J. 2006;20(6):779-81.
80. Islam LN, Nabi AH, Rahman MM, Zahid MS. Association of respiratory complications and elevated serum immunoglobulins with drinking water arsenic toxicity in human. J Environ Sci Health A Tox Hazard Subst Environ Eng. 2007;42(12):1807-14.
81. Islam LN, Zahid MS, Nabi AH, Hossain M. Function of serum complement in drinking water arsenic toxicity. J Toxicol. 2012;2012:302817.
82. Ferrario D. In vitro Assessment of Arsenic Immune Toxicity using Human Cord Blood and Murine Bone Marrow Cells: University of Konstanz; 2009.
83. Selgrade MK. Immunotoxicity: the risk is real. Toxicol Sci. 2007;100(2):328-32.
84. Soria EA, Perez RD, Queralt I, Perez CA, Bongiovanni GA. Immunotoxicological effects of arsenic bioaccumulation on spatial metallomics and cellular enzyme response in the spleen of male Wistar rats after oral intake. Toxicol Lett. 2017;266:65-73.
85. Lemarie A, Morzadec C, Bourdonnay E, Fardel O, Vernhet L. Human macrophages constitute targets for immunotoxic inorganic arsenic. J Immunol. 2006;177(5):3019-27.
86. Morcillo P, Cordero H, Meseguer J, Esteban MA, Cuesta A. In vitro immunotoxicological effects of heavy metals on European sea bass (Dicentrarchus labrax L.) head-kidney leucocytes. Fish Shellfish Immunol. 2015;47(1):245-54.
87. Alberts B JA, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cell. 4th ed. New York: Garland Science; 2002.
88. Blackburn EH, Epel ES, Lin J. Human telomere biology: A contributory and interactive factor in aging, disease risks, and protection. Science. 2015;350(6265):1193-8.
89. Barrett JH, Iles MM, Dunning AM, Pooley KA. Telomere length and common disease: study design and analytical challenges. Hum Genet. 2015;134(7):679-89.
90. Gianesin K, Noguera-Julian A, Zanchetta M, Del Bianco P, Petrara MR, Freguja R, et al. Premature aging and immune senescence in HIV-infected children. AIDS. 2016;30(9):1363-73.
91. Zhou M, Zhu L, Cui X, Feng L, Zhao X, He S, et al. Influence of diet on leukocyte telomere length, markers of inflammation and oxidative stress in individuals with varied glucose tolerance: a Chinese population study. Nutr J. 2016;15:39.
92. Li H, Hedmer M, Wojdacz T, Hossain MB, Lindh CH, Tinnerberg H, et al. Oxidative stress, telomere shortening, and DNA methylation in relation to low-to-moderate occupational exposure to welding fumes. Environ Mol Mutagen. 2015;56(8):684-93.

93. Zhang X, Zhao Q, Zhu W, Liu T, Xie SH, Zhong LX, et al. The Association of Telomere Length in Peripheral Blood Cells with Cancer Risk: A Systematic Review and Meta-analysis of Prospective Studies. Cancer Epidemiol Biomarkers Prev. 2017;26(9):1381-90.
94. Ameer SS, Xu Y, Engstrom K, Li H, Tallving P, Nermell B, et al. Exposure to Inorganic Arsenic Is Associated with Increased Mitochondrial DNA Copy Number and Longer Telomere Length in Peripheral Blood. Front Cell Dev Biol. 2016;4:87.
95. Gao J, Roy S, Tong L, Argos M, Jasmine F, Rahaman R, et al. Arsenic exposure, telomere length, and expression of telomere-related genes among Bangladeshi individuals. Environ Res. 2015;136:462-9.
96. Li H, Engstrom K, Vahter M, Broberg K. Arsenic exposure through drinking water is associated with longer telomeres in peripheral blood. Chem Res Toxicol. 2012;25(11):2333-9.
97. SEARO. Regional Nutrition Strategy: Addressing malnutrition and micronutrient deficiencies (2011-2015).
98. De Benoist B. Worldwide prevalence of anaemia 1993-2005 of WHO Global Database of anaemia. Geneva: World Health Organization, Centers for Disease Control and Prevention (U.S.); 2008.
99. Allen LH. Anemia and iron deficiency: effects on pregnancy outcome. Am J Clin Nutr. 2000;71(5 Suppl):1280S-4S.
100. WHO. Serum retinol concentrations for determining the prevalence of vitamin A deficiency in populations. Vitamin and Mineral Nutrition Information System. Geneva; 2011.
101. WHO. Anaemia. 2015.
102. WHO. Centers for Disease Control and Prevention. Assessing the iron status of populations. Geneva, Switzerland; 2004.
103. Finch CA, Bellotti V, Stray S, Lipschitz DA, Cook JD, Pippard MJ, et al. Plasma ferritin determination as a diagnostic tool. West J Med. 1986;145(5):657-63.
104. Peyrin-Biroulet L, Williet N, Cacoub P. Guidelines on the diagnosis and treatment of iron deficiency across indications: a systematic review. Am J Clin Nutr. 2015;102(6):1585-94.
105. Berlin T, Meyer A, Rotman-Pikielny P, Natur A, Levy Y. Soluble transferrin receptor as a diagnostic laboratory test for detection of iron deficiency anemia in acute illness of hospitalized patients. Isr Med Assoc J. 2011;13(2):96-8.
106. Goodnough LT, Nemeth E, Ganz T. Detection, evaluation, and management of iron-restricted erythropoiesis. Blood. 2010;116(23):4754-61.
107. Lozoff B, Beard J, Connor J, Barbara F, Georgieff M, Schallert T. Long-lasting neural and behavioral effects of iron deficiency in infancy. Nutr Rev. 2006;64(5 Pt 2):S34-43; discussion S72-91.
108. Roncagliolo M, Garrido M, Walter T, Peirano P, Lozoff B. Evidence of altered central nervous system development in infants with iron deficiency anemia at 6 mo: delayed maturation of auditory brainstem responses. Am J Clin Nutr. 1998;68(3):683-90.
109. Ergul AB, Turanoglu C, Karakukcu C, Kazanci EG, Torun YA. Increased Vitamin B12 Levels in Children with Zinc Deficiency. Int J Vitam Nutr Res. 2018:1-6.
110. Bailey RL, West KP, Jr., Black RE. The epidemiology of global micronutrient deficiencies. Ann Nutr Metab. 2015;66 Suppl 2:22-33.
111. Fischer Walker C, Black RE. Zinc and the risk for infectious disease. Annu Rev Nutr. 2004;24:255-75.
112. Balarajan Y, Ramakrishnan U, Ozaltin E, Shankar AH, Subramanian SV. Anaemia in low-income and middle-income countries. Lancet. 2011;378(9809):2123-35.

113. Ohrvik VE, Witthoft CM. Human folate bioavailability. Nutrients. 2011;3(4):475-90.
114. Strand TA, Taneja S, Bhandari N, Refsum H, Ueland PM, Gjessing HK, et al. Folate, but not vitamin B-12 status, predicts respiratory morbidity in north Indian children. Am J Clin Nutr. 2007;86(1):139-44.
115. Graham SM, Arvela OM, Wise GA. Long-term neurologic consequences of nutritional vitamin B12 deficiency in infants. J Pediatr. 1992;121(5 Pt 1):710-4.
116. Rasmussen SA, Fernhoff PM, Scanlon KS. Vitamin B12 deficiency in children and adolescents. J Pediatr. 2001;138(1):10-7.
117. Semba RD, Bloem MW. The anemia of vitamin A deficiency: epidemiology and pathogenesis. Eur J Clin Nutr. 2002;56(4):271-81.
118. Brown CC, Noelle RJ. Seeing through the dark: New insights into the immune regulatory functions of vitamin A. Eur J Immunol. 2015;45(5):1287-95.
119. NMS. National Micronutrients Status Survey 2011–12. ICDDR,B, UNICEF, Bangladesh, GAIN and Institute of Public Health and Nutrition; 2013.
120. Biswas D, Banerjee M, Sen G, Das JK, Banerjee A, Sau TJ, et al. Mechanism of erythrocyte death in human population exposed to arsenic through drinking water. Toxicol Appl Pharmacol. 2008;230(1):57-66.
121. Heck JE, Chen Y, Grann VR, Slavkovich V, Parvez F, Ahsan H. Arsenic exposure and anemia in Bangladesh: a population-based study. J Occup Environ Med. 2008;50(1):80-7.
122. Hopenhayn C, Bush HM, Bingcang A, Hertz-Picciotto I. Association between arsenic exposure from drinking water and anemia during pregnancy. J Occup Environ Med. 2006;48(6):635-43.
123. Majumdar KK, Guha Mazumder DN, Ghose N, Ghose A, Lahiri S. Systemic manifestations in chronic arsenic toxicity in absence of skin lesions in West Bengal. Indian J Med Res. 2009;129(1):75-82.
124. Parvez F, Medina S, Santella RM, Islam T, Lauer FT, Alam N, et al. Arsenic exposures alter clinical indicators of anemia in a male population of smokers and non-smokers in Bangladesh. Toxicol Appl Pharmacol. 2017;331:62-8.
125. Kyle RA, Pease GL. Hematologic Aspects of Arsenic Intoxication. N Engl J Med. 1965;273:18-23.
126. Lerman BB, Ali N, Green D. Megaloblastic, dyserythropoietic anemia following arsenic ingestion. Ann Clin Lab Sci. 1980;10(6):515-7.
127. Flora SJ, Bhadauria S, Pant SC, Dhaked RK. Arsenic induced blood and brain oxidative stress and its response to some thiol chelators in rats. Life Sci. 2005;77(18):2324-37.
128. Vigeh M, Yokoyama K, Matsukawa T, Shinohara A, Ohtani K. The relation of maternal blood arsenic to anemia during pregnancy. Women Health. 2015;55(1):42-57.
129. Harrington JM, Middaugh JP, Morse DL, Housworth J. A survey of a population exposed to high concentrations of arsenic in well water in Fairbanks, Alaska. Am J Epidemiol. 1978;108(5):377-85.
130. Merrill RD, Shamim AA, Ali H, Labrique AB, Schulze K, Christian P, et al. High prevalence of anemia with lack of iron deficiency among women in rural Bangladesh: a role for thalassemia and iron in groundwater. Asia Pac J Clin Nutr. 2012;21(3):416-24.
131. Southwick JW, AE; Beck, MM; Whitley, T; Isaacs, R; Petajan, J; Hansen, CD. An epidemiological study of arsenic in drinking water in Millard County, Utah. New York: Van Nostrand Reinhold Company; 1983.

132. Breton CV, Houseman EA, Kile ML, Quamruzzaman Q, Rahman M, Mahiuddin G, et al. Gender-specific protective effect of hemoglobin on arsenic-induced skin lesions. Cancer Epidemiol Biomarkers Prev. 2006;15(5):902-7.
133. Hatlelid KM, Brailsford C, Carter DE. An in vitro model for arsine toxicity using isolated red blood cells. Fundam Appl Toxicol. 1995;25(2):302-6.
134. Hatlelid KM, Carter DE. Reactive oxygen species do not cause arsine-induced hemoglobin damage. J Toxicol Environ Health. 1997;50(5):463-74.
135. Winski SL, Barber DS, Rael LT, Carter DE. Sequence of toxic events in arsine-induced hemolysis in vitro: implications for the mechanism of toxicity in human erythrocytes. Fundam Appl Toxicol. 1997;38(2):123-8.
136. Winski SL, Carter DE. Interactions of rat red blood cell sulfhydryls with arsenate and arsenite. J Toxicol Environ Health. 1995;46(3):379-97.
137. Winski SL, Carter DE. Arsenate toxicity in human erythrocytes: characterization of morphologic changes and determination of the mechanism of damage. J Toxicol Environ Health A. 1998;53(5):345-55.
138. Zhang TL, Gao YX, Lu JF, Wang K. Arsenite, arsenate and vanadate affect human erythrocyte membrane. J Inorg Biochem. 2000;79(1-4):195-203.
139. Lu M, Wang H, Li XF, Lu X, Cullen WR, Arnold LL, et al. Evidence of hemoglobin binding to arsenic as a basis for the accumulation of arsenic in rat blood. Chem Res Toxicol. 2004;17(12):1733-42.
140. Shiobara Y, Ogra Y, Suzuki KT. Animal species difference in the uptake of dimethylarsinous acid (DMA(III)) by red blood cells. Chem Res Toxicol. 2001;14(10):1446-52.
141. Naranmandura H, Suzuki KT. Identification of the major arsenic-binding protein in rat plasma as the ternary dimethylarsinous-hemoglobin-haptoglobin complex. Chem Res Toxicol. 2008;21(3):678-85.
142. Berglund M, Lindberg AL, Rahman M, Yunus M, Grander M, Lonnerdal B, et al. Gender and age differences in mixed metal exposure and urinary excretion. Environ Res. 2011;111(8):1271-9.
143. Musimwa AM, Kanteng GW, Kitoko HT, Luboya ON. [Trace elements in serum of malnourished and well-nourished children living in Lubumbashi and Kawama]. Pan Afr Med J. 2016;24:11.
144. Peraza MA, Ayala-Fierro F, Barber DS, Casarez E, Rael LT. Effects of micronutrients on metal toxicity. Environ Health Perspect. 1998;106 Suppl 1:203-16.
145. Xu Y, Wang H, Wang Y, Zheng Y, Sun G. Effects of folate on arsenic toxicity in Chang human hepatocytes: involvement of folate antioxidant properties. Toxicol Lett. 2010;195(1):44-50.
146. Valentine JL, Cebrian ME, Garcia-vargas GG, Faraji B, Kuo J, Gibb HJ, et al. Daily selenium intake estimates for residents of arsenic-endemic areas. Environ Res. 1994;64(1):1-9.
147. Kreppel H, Liu J, Liu Y, Reichl FX, Klaassen CD. Zinc-induced arsenite tolerance in mice. Fundam Appl Toxicol. 1994;23(1):32-7.
148. Gardner R, Hamadani J, Grander M, Tofail F, Nermell B, Palm B, et al. Persistent exposure to arsenic via drinking water in rural Bangladesh despite major mitigation efforts. Am J Public Health. 2011;101 Suppl 1:S333-8.
149. icddrb. International Centre for Diarrhoeal Disease Research Bangladesh. Health and Demographic Surveillance System-Matlab. Registration of Health and Demographic Events 2004. Dhaka, Bangladesh icddr,b; 2006.

150. Rahman M, Vahter M, Wahed MA, Sohel N, Yunus M, Streatfield PK, et al. Prevalence of arsenic exposure and skin lesions. A population based survey in Matlab, Bangladesh. J Epidemiol Community Health. 2006;60(3):242-8.
151. Vahter ME, Li L, Nermell B, Rahman A, El Arifeen S, Rahman M, et al. Arsenic exposure in pregnancy: a population-based study in Matlab, Bangladesh. J Health Popul Nutr. 2006;24(2):236-45.
152. Nahian MA. Matlab HDSS Arsenic Screening Survey 2013/14. Gobeshona Monthly Seminar. icddr,b; 2016.
153. Nasreen S, Luby SP, Brooks WA, Homaira N, Al Mamun A, Bhuiyan MU, et al. Population-based incidence of severe acute respiratory virus infections among children aged <5 years in rural Bangladesh, June-October 2010. PLoS One. 2014;9(2):e89978.
154. Persson LA, Arifeen S, Ekstrom EC, Rasmussen KM, Frongillo EA, Yunus M, et al. Effects of prenatal micronutrient and early food supplementation on maternal hemoglobin, birth weight, and infant mortality among children in Bangladesh: the MINIMat randomized trial. JAMA. 2012;307(19):2050-9.
155. Mannan T, Ahmed S, Akhtar E, Roy AK, Haq MA, Roy A, et al. Maternal Micronutrient Supplementation and Long Term Health Impact in Children in Rural Bangladesh. PLoS One. 2016;11(8):e0161294.
156. Mannan T, Ahmed S, Akhtar E, Ahsan KB, Haq A, Kippler M, et al. Associations of Arsenic Exposure With Telomere Length and Naive T Cells in Childhood-A Birth Cohort Study. Toxicol Sci. 2018;164(2):539-49.
157. Ekstrom EC, Lindstrom E, Raqib R, El Arifeen S, Basu S, Brismar K, et al. Effects of prenatal micronutrient and early food supplementation on metabolic status of the offspring at 4.5 years of age. The MINIMat randomized trial in rural Bangladesh. Int J Epidemiol. 2016;45(5):1656-67.
158. Svefors P, Selling KE, Shaheen R, Khan AI, Persson LA, Lindholm L. Cost-effectiveness of prenatal food and micronutrient interventions on under-five mortality and stunting: Analysis of data from the MINIMat randomized trial, Bangladesh. PLoS One. 2018;13(2):e0191260.
159. Hawlader MD, Ma E, Noguchi E, Itoh M, Arifeen SE, Persson LA, et al. Ascaris lumbricoids Infection as a Risk Factor for Asthma and Atopy in Rural Bangladeshi Children. Trop Med Health. 2014;42(2):77-85.
160. Hawlader MD, Noguchi E, El Arifeen S, Persson LA, Moore SE, Raqib R, et al. Nutritional status and childhood wheezing in rural Bangladesh. Public Health Nutr. 2014;17(7):1570-7.
161. Ahmed S, Rekha RS, Ahsan KB, Doi M, Grander M, Roy AK, et al. Arsenic exposure affects plasma insulin-like growth factor 1 (IGF-1) in children in rural Bangladesh. PLoS One. 2013;8(11):e81530.
162. Doi M, Rekha RS, Ahmed S, Okada M, Roy AK, El Arifeen S, et al. Association between calcium in cord blood and newborn size in Bangladesh. Br J Nutr. 2011;106(9):1398-407.
163. de Onis M, Onyango AW, Borghi E, Siyam A, Nishida C, Siekmann J. Development of a WHO growth reference for school-aged children and adolescents. Bull World Health Organ. 2007;85(9):660-7.
164. Gwatkin DR, Rustein S, Johnson K, Pande RP, Wagstaff A. Socioeconomic differences in health, nutrition, and population in Bangladesh, HNP/Poverty Thematic Group Working Paper. Washington, DC: The World Bank; 2000.

165. Kolbe-Busch S, Lotz J, Hafner G, Blanckaert NJ, Claeys G, Togni G, et al. Multicenter evaluation of a fully mechanized soluble transferrin receptor assay on the Hitachi and cobas integra analyzers. the determination of reference ranges. Clin Chem Lab Med. 2002;40(5):529-36.
166. Akcam M, Ozdem S, Yilmaz A, Gultekin M, Artan R. Serum ferritin, vitamin B(12), folate, and zinc levels in children infected with Helicobacter pylori. Dig Dis Sci. 2007;52(2):405-10.
167. IZiNCG. International Zinc Nutrition Collaborative Group. Assessing population zinc status with serum zinc concentration. IZiNCG Technical Brief No. 02. 2012.
168. Diaz OP, Arcos R, Tapia Y, Pastene R, Velez D, Devesa V, et al. Estimation of arsenic intake from drinking water and food (raw and cooked) in a rural village of northern Chile. Urine as a biomarker of recent exposure. Int J Environ Res Public Health. 2015;12(5):5614-33.
169. Nermell B, Lindberg AL, Rahman M, Berglund M, Persson LA, El Arifeen S, et al. Urinary arsenic concentration adjustment factors and malnutrition. Environ Res. 2008;106(2):212-8.
170. Raqib R, Alam DS, Sarker P, Ahmad SM, Ara G, Yunus M, et al. Low birth weight is associated with altered immune function in rural Bangladeshi children: a birth cohort study. Am J Clin Nutr. 2007;85(3):845-52.
171. Ngom PT, Collinson AC, Pido-Lopez J, Henson SM, Prentice AM, Aspinall R. Improved thymic function in exclusively breastfed infants is associated with higher interleukin 7 concentrations in their mothers' breast milk. Am J Clin Nutr. 2004;80(3):722-8.
172. O'Callaghan NJ, Fenech M. A quantitative PCR method for measuring absolute telomere length. Biol Proced Online. 2011;13:3.
173. Yamauchi H, Aminaka Y, Yoshida K, Sun G, Pi J, Waalkes MP. Evaluation of DNA damage in patients with arsenic poisoning: urinary 8-hydroxydeoxyguanine. Toxicol Appl Pharmacol. 2004;198(3):291-6.
174. Sdogou T, Tsentidis C, Gourgiotis D, Marmarinos A, Gkourogianni A, Papassotiriou I, et al. Immunoassay-based serum hepcidin reference range measurements in healthy children: differences among age groups. J Clin Lab Anal. 2015;29(1):10-4.
175. NHANES.CDC. Laboratory Procedure Manual. Complete Blood Count (CBC) with Five-Part Differential. 2003-2004.
176. Manley SA, George GN, Pickering IJ, Glass RS, Prenner EJ, Yamdagni R, et al. The seleno bis(S-glutathionyl) arsinium ion is assembled in erythrocyte lysate. Chem Res Toxicol. 2006;19(4):601-7.
177. Surdu S, Bloom MS, Neamtiu IA, Pop C, Anastasiu D, Fitzgerald EF, et al. Consumption of arsenic-contaminated drinking water and anemia among pregnant and non-pregnant women in northwestern Romania. Environ Res. 2015;140:657-60.
178. Islam LN, Nabi AH, Rahman MM, Khan MA, Kazi AI. Association of clinical complications with nutritional status and the prevalence of leukopenia among arsenic patients in Bangladesh. Int J Environ Res Public Health. 2004;1(2):74-82.
179. Kile ML, Faraj JM, Ronnenberg AG, Quamruzzaman Q, Rahman M, Mostofa G, et al. A cross sectional study of anemia and iron deficiency as risk factors for arsenic-induced skin lesions in Bangladeshi women. BMC Public Health. 2016;16:158.
180. Lopez-Rodriguez G, Galvan M, Gonzalez-Unzaga M, Hernandez Avila J, Perez-Labra M. Blood toxic metals and hemoglobin levels in Mexican children. Environ Monit Assess. 2017;189(4):179.
181. Rosado JL, Ronquillo D, Kordas K, Rojas O, Alatorre J, Lopez P, et al. Arsenic exposure and cognitive performance in Mexican schoolchildren. Environ Health Perspect. 2007;115(9):1371-5.

182. Dey NC, Ali AM. Changes in the Use of Safe Water and Water Safety Measures in Water, Sanitation and Hygiene Intervention Areas of Bangladesh: A Midline Assessment. Research and Evaluation Division, BRAC; 2011.
183. Gamble MV, Liu X, Ahsan H, Pilsner JR, Ilievski V, Slavkovich V, et al. Folate and arsenic metabolism: a double-blind, placebo-controlled folic acid-supplementation trial in Bangladesh. Am J Clin Nutr. 2006;84(5):1093-101.
184. Adams AM, Ahmed R, Latif AH, Rasheed S, Das SK, Hasib E, et al. Impact of fortified biscuits on micronutrient deficiencies among primary school children in Bangladesh. PLoS One. 2017;12(4):e0174673.
185. WHO. Global Database on Anaemia. Bangladesh. Vitamin and Mineral Nutrition Information System (VMNIS). 2007.
186. BBS/UNICEF. Anemia prevalence survey of Urban Bangladesh and Rural Chittagong Hill Tracts.; 2003.
187. HKI/IPHN. Anaemia is a serious public health problem in pre-school children and pregnant women in rural Bangladesh. 2002. Report No.: NSP Bull No10.
188. Hossain D, Islam MS, Sultana N. Assessment of Iron Contamination in Groundwater at Tangail Municipality, Bangladesh. J Environ Sci & Natural Resources 2013;6:117 - 21.
189. Kinniburgh DG, Smedley PL. Arsenic contamination of groundwater in Bangladesh. UK: Keyworth; 2001.
190. Hossain M, Bhattacharya P, Rahman M. Water quality of drinking water well samples, Matlab, Bangladesh. Geochemistry of Arsenic and Other Toxic Elements and Assessment of Environmental Risks in Global Groundwater Systems II; Oregon Convention Center. Portland: Geological Society of America; 2009.
191. Bhattacharya P, Hossain M, Ahmed KM. Baseline Water Quality of 243 Drinking Water Tubewells Installed at Intermediate Deep Aquifer (IDA) in Matlab, Southeastern Bangladesh. Groundwater and Surface-Water Arsenic: From Source to Sink II; 22 October 2014; Vancouver, British Columbia, Canada.2014. p. 811.
192. Merrill RD, Shamim AA, Ali H, Jahan N, Labrique AB, Schulze K, et al. Iron status of women is associated with the iron concentration of potable groundwater in rural Bangladesh. J Nutr. 2011;141(5):944-9.
193. United, Nations. Millenium Development Goals and beyond 2015. 2013.
194. NIPORT. National Institute of Population Research and Training, MaA, and ICF International. Bangladesh Demographic and Health Survey 2014: Key Indicators. Dhaka, Bangladesh.; 2015.
195. Das SK, Chisti MJ, Malek MA, Das J, Salam MA, Ahmed T, et al. Changing childhood malnutrition in Bangladesh: trends over the last two decades in urban-rural differentials (1993-2012). Public Health Nutr. 2015;18(10):1718-27.
196. Henriksson H. Prevalence of anemia and its association with socio-demographic factors and micronutrient deficiencies in 4.5-year old children in Matlab, Bangladesh: a cross-sectional follow-up study. Uppsala: Uppsala University; 2015.
197. Janus J, Moerschel SK. Evaluation of anemia in children. Am Fam Physician. 2010;81(12):1462-71.
198. Rosales FJ, Jang JT, Pinero DJ, Erikson KM, Beard JL, Ross AC. Iron deficiency in young rats alters the distribution of vitamin A between plasma and liver and between hepatic retinol and retinyl esters. J Nutr. 1999;129(6):1223-8.

199. Abioye AI, Aboud S, Premji Z, Etheredge AJ, Gunaratna NS, Sudfeld CR, et al. Iron Supplementation Affects Hematologic Biomarker Concentrations and Pregnancy Outcomes among Iron-Deficient Tanzanian Women. J Nutr. 2016;146(6):1162-71.
200. Shamim AA, Kabir A, Merrill RD, Ali H, Rashid M, Schulze K, et al. Plasma zinc, vitamin B(12) and alpha-tocopherol are positively and plasma gamma-tocopherol is negatively associated with Hb concentration in early pregnancy in north-west Bangladesh. Public Health Nutr. 2013;16(8):1354-61.
201. Silva Neto LGR, Santos Neto JED, Bueno NB, de Oliveira SL, Ataide TDR. Effects of iron supplementation versus dietary iron on the nutritional iron status: Systematic review with meta-analysis of randomized controlled trials. Crit Rev Food Sci Nutr. 2018:1-9.
202. Khan AI, Kabir I, Ekstrom EC, Asling-Monemi K, Alam DS, Frongillo EA, et al. Effects of prenatal food and micronutrient supplementation on child growth from birth to 54 months of age: a randomized trial in Bangladesh. Nutr J. 2011;10:134.
203. Lu WP, Lu MS, Li ZH, Zhang CX. Effects of multimicronutrient supplementation during pregnancy on postnatal growth of children under 5 years of age: a meta-analysis of randomized controlled trials. PLoS One. 2014;9(2):e88496.
204. Hanieh S, Ha TT, Simpson JA, Braat S, Thuy TT, Tran TD, et al. Effect of low-dose versus higher-dose antenatal iron supplementation on child health outcomes at 36 months of age in Viet Nam: longitudinal follow-up of a cluster randomised controlled trial. BMJ Glob Health. 2017;2(3):e000368.
205. Khavari N, Jiang H, Manji K, Msamanga G, Spiegelman D, Fawzi W, et al. Maternal multivitamin supplementation reduces the risk of diarrhoea among HIV-exposed children through age 5 years. Int Health. 2014;6(4):298-305.
206. Palmer AC, Schulze KJ, Khatry SK, De Luca LM, West KP, Jr. Maternal vitamin A supplementation increases natural antibody concentrations of preadolescent offspring in rural Nepal. Nutrition. 2015;31(6):813-9.
207. Capra L, Tezza G, Mazzei F, Boner AL. The origins of health and disease: the influence of maternal diseases and lifestyle during gestation. Ital J Pediatr. 2013;39:7.
208. Demirchyan A, Petrosyan V, Sargsyan V, Hekimian K. Prevalence and determinants of anaemia among children aged 0-59 months in a rural region of Armenia: a case-control study. Public Health Nutr. 2016;19(7):1260-9.
209. Subramanian SV, Ackerson LK, Davey Smith G, John NA. Association of maternal height with child mortality, anthropometric failure, and anemia in India. JAMA. 2009;301(16):1691-701.
210. Zhao A, Zhang Y, Peng Y, Li J, Yang T, Liu Z, et al. Prevalence of anemia and its risk factors among children 6-36 months old in Burma. Am J Trop Med Hyg. 2012;87(2):306-11.
211. Kaati G, Bygren LO, Pembrey M, Sjostrom M. Transgenerational response to nutrition, early life circumstances and longevity. Eur J Hum Genet. 2007;15(7):784-90.
212. Borghini A, Faita F, Mercuri A, Minichilli F, Bustaffa E, Bianchi F, et al. Arsenic exposure, genetic susceptibility and leukocyte telomere length in an Italian young adult population. Mutagenesis. 2016;31(5):539-46.
213. Chatterjee D, Bhattacharjee P, Sau TJ, Das JK, Sarma N, Bandyopadhyay AK, et al. Arsenic exposure through drinking water leads to senescence and alteration of telomere length in humans: A case-control study in West Bengal, India. Mol Carcinog. 2015;54(9):800-9.
214. Romano GH, Harari Y, Yehuda T, Podhorzer A, Rubinstein L, Shamir R, et al. Environmental stresses disrupt telomere length homeostasis. PLoS Genet. 2013;9(9):e1003721.

215. Zhang X, Lin S, Funk WE, Hou L. Environmental and occupational exposure to chemicals and telomere length in human studies. Occup Environ Med. 2013;70(10):743-9.
216. Zhang TC, Schmitt MT, Mumford JL. Effects of arsenic on telomerase and telomeres in relation to cell proliferation and apoptosis in human keratinocytes and leukemia cells in vitro. Carcinogenesis. 2003;24(11):1811-7.
217. Chou WC, Hawkins AL, Barrett JF, Griffin CA, Dang CV. Arsenic inhibition of telomerase transcription leads to genetic instability. J Clin Invest. 2001;108(10):1541-7.
218. Hackett JA, Greider CW. Balancing instability: dual roles for telomerase and telomere dysfunction in tumorigenesis. Oncogene. 2002;21(4):619-26.
219. Mackall CL, Gress RE. Thymic aging and T-cell regeneration. Immunol Rev. 1997;160:91-102.
220. Douek DC, Koup RA, McFarland RD, Sullivan JL, Luzuriaga K. Effect of HIV on thymic function before and after antiretroviral therapy in children. J Infect Dis. 2000;181(4):1479-82.
221. Zhang L, Lewin SR, Markowitz M, Lin HH, Skulsky E, Karanicolas R, et al. Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy. J Exp Med. 1999;190(5):725-32.
222. Gul KA, Sonerud T, Fjaerli HO, Nakstad B, Abrahamsen TG, Inchley CS. Thymus activity measured by T-cell receptor excision circles in patients with different severities of respiratory syncytial virus infection. BMC Infect Dis. 2017;17(1):18.
223. Sandgaard KS, Lewis J, Adams S, Klein N, Callard R. Antiretroviral therapy increases thymic output in children with HIV. AIDS. 2014;28(2):209-14.
224. Ye P, Kirschner DE, Kourtis AP. The thymus during HIV disease: role in pathogenesis and in immune recovery. Curr HIV Res. 2004;2(2):177-83.
225. Torrao RC, Bennett SJ, Brown JE, Griffiths HR. Does metabolic reprogramming underpin age-associated changes in T cell phenotype and function? Free Radic Biol Med. 2014;71:26-35.
226. Benetos A, Kark JD, Susser E, Kimura M, Sinnreich R, Chen W, et al. Tracking and fixed ranking of leukocyte telomere length across the adult life course. Aging Cell. 2013;12(4):615-21.
227. Daniali L, Benetos A, Susser E, Kark JD, Labat C, Kimura M, et al. Telomeres shorten at equivalent rates in somatic tissues of adults. Nat Commun. 2013;4:1597.
228. Liu L, Trimarchi JR, Navarro P, Blasco MA, Keefe DL. Oxidative stress contributes to arsenic-induced telomere attrition, chromosome instability, and apoptosis. J Biol Chem. 2003;278(34):31998-2004.
229. Singh AP, Goel RK, Kaur T. Mechanisms pertaining to arsenic toxicity. Toxicol Int. 2011;18(2):87-93.