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ELEVATED DRINKING WATER MANGANESE AND FETAL AND CHILD HEALTH AND DEVELOPMENT

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Cover picture 'Mn: manganese and mother-newborn' by Nazifa Tasnim and Ahsan Nawroj

Elevated drinking water manganese and fetal and child health and development
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my wife, Emily, my beloved children, Farsan and Fahman, and my parents

ABSTRACT

Manganese (Mn) is an essential element that functions as a cofactor in the metabolism of carbohydrates and proteins, and it is also incorporated in antioxidants such as superoxide dismutase. In general, intake of Mn mainly occurs via food. Additional or excess exposure might occur via drinking water, as elevated concentrations of Mn in ground water are prevalent worldwide. In recent years, several studies have shown associations between Mn concentrations in drinking water and adverse health effects in children. A few studies have concerned early-life exposure, but these have mostly been cross sectional in design or with a very short follow-up of the children. Prospective studies which follow the children from early intrauterine life to childhood and explore associated adverse effects are lacking.

The overall aim of the present thesis was to assess the potential effects of Mn exposure via drinking water during pregnancy and early-life on fetal outcomes (spontaneous abortion and perinatal mortality), size at birth, and child development (cognitive function and behavior). These prospective cohort studies were nested in a population-based trial involving food and micronutrient supplementation (Maternal and Infant Nutrition Interventions in Matlab; MINIMat), conducted from November 2001 to October 2003, in 4,436 pregnant women in Matlab, Bangladesh. A subsample of the children born within the MINIMat trial was selected for longitudinal follow-up of growth and development until 10 years of age. In Matlab, 70% of the drinking water wells contained high concentration of arsenic (>10 µg/L, mostly in shallow wells), a potent toxicant and carcinogen. To reduce the exposure to arsenic the inhabitants were recommended to install deeper wells. Later we revealed that over 40% of the wells (mainly deeper well >50 m), contained >400 µg Mn/L (previous WHO guideline value). The Mn exposure in the present studies was assessed by the concentrations in drinking water used by the mothers during pregnancy and by the children at 5 and 10 years of age, and measured by inductively coupled plasma mass spectrometry.

The median water Mn concentration has increased from about 200 µg/L during pregnancy to 339 µg/L when the children were 10 years old, whereas the arsenic concentrations decreased (median from 33 to 2.3 µg/L) during the same period. This is probably due to the ongoing installation of deeper wells. In early pregnancy (n=1,875), women in the highest tertile of water Mn concentrations (median=1,292 µg/L) had an approximately 35% reduced risk (Odds Ratio = 0.65, 95% CI 0.43, 0.99) of spontaneous abortion, compared with women in the lowest tertile (median=56 µg/L). This is possibly related to the role of Mn in the placental antioxidant defense. Elevated water Mn concentrations were not related to any increased risk of perinatal mortality.

The newborns (n=1,177) to mothers in the highest tertile of water Mn (median=1,495 µg/L) were on average 0.5 cm shorter (0.20 SD) compared to those in the lowest tertile (median 56 µg/L). The association was strongest in the girls, but apparent also in the boys of mothers with low hemoglobin (Hb) values (<114 g/L), likely due to higher absorption of Mn at low iron status. The findings indicate that elevated levels of Mn in drinking water during pregnancy may impair fetal growth. The association between erythrocyte Mn concentrations and size at birth was less apparent.

The prenatal Mn exposure, but not the postnatal, was associated with aggravation of the difficult behavior at 10 years of age (n=1,295). For each increase of one mg Mn/L, the difficult behavior scores increased by 0.5 points (0.13 SD) in boys and 0.7 points (0.18 SD) in girls of mothers with anemia (Hb<110 g/L). Associations of water Mn (especially at 5 years) with boys´ cognitive function were generally inverse (effect size ~0.15 SD per mg/L of water Mn). In girls, there was a positive association of maternal water Mn below 3 mg/L with cognitive function and pro-social behavior. However, a tendency of inverse associations with cognitive function were observed at higher Mn concentrations (>3 mg/L) and in girls of mothers with anemia. Early-life appeared to be a particularly susceptible period to inverse effects on development by elevated water Mn concentrations, although the effect differed by outcome, time-point of exposure and child gender.

In conclusion, the Mn concentrations in well water varied widely. Elevated concentrations during pregnancy appeared to be protective for early fetal loss. On the other hand, the exposure during pregnancy decreased fetal growth and impaired the children´s cognitive function and worsened their difficult behavior, especially in children of anemic women. Thus, Mn concentration in drinking water is an important public health concern world-wide. However, in arsenic contaminated areas, the benefit of deep wells with less arsenic is most likely higher than the modest adverse effects of Mn. In order to reduce the exposure to arsenic and Mn through well water, screening for both elements by sediment color tools could be done during installation of new wells, as well as digging wells in aquifers that are low in both arsenic and Mn (deeper than 100 m). As Mn absorption tends to be higher in anemic women, existing routine programs, including assessment of anemia during pregnancy and supplementation with iron and folic acid, should be strengthened. Also, screening and treating children with anemia can be proposed as an important public health intervention.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numbers I-III.

I. **Rahman SM,** Åkesson A, Kippler M, Grandér M, Hamadani JD, Streatfield PK, Persson LÅ, El Arifeen S, Vahter M. Elevated manganese concentrations in drinking water may be beneficial for fetal survival. *PLoS One. 16;8(9):e74119, 2013.*

II. **Rahman SM,** Kippler M, Ahmed S, Palm B, El Arifeen S, Vahter M. Manganese exposure through drinking water during pregnancy and size at birth: A prospective cohort study. *Reprod Toxicol. 2015 Mar 28;53:68-74 [Epub ahead of print].*

III. **Rahman SM,** Kippler M, Tofail F, Nordqvist H, Hamadani JD, Vahter M. Critical window of manganese exposure through drinking water for child IQ and behavior at 10 years of age: A prospective cohort study. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

AsMat	Arsenic study at Matlab
BMI	Body mass index
DMT1	Divalent metal transporter 1
GW	Gestational week
HDSS	Health and Demographic Surveillance System
ICD-10	International Classification of Disease, Disease Injuries and Cause of Death
icddr,b	International Centre for Diarrhoeal Disease Research in Bangladesh
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
MINIMat	Maternal and Infant Nutrition Interventions in Matlab
MnSOD	Manganese superoxide dismutase
SES	Socioeconomic status
LOD	Limit of detection
Hb	Hemoglobin
IQ	Intelligent quotient
SDQ	Strengths and difficulties questionnaire
VCI	Verbal comprehension index
PRI	Perceptual reasoning index
WMI	Working memory index
PSI	Processing speed index
FSIQ	Full scale IQ
HOME	Home observation for measurement of environment
HAZ	Height-for-age Z score

1 INTRODUCTION

This thesis includes studies on maternal and early-life exposure to manganese (Mn) and effects on fetal growth and child development. Manganese is essential for a variety of physiological processes, including amino acid, lipid, protein, and carbohydrate metabolism (Erikson et al. 2007). On the other hand, excess Mn exposure during early life has been associated with adverse health effects such as neurotoxicity (Menezes-Filho et al. 2009; Roels et al. 2012), but the most critical window of early-life Mn exposure is not known. Thus, it is important to study Mn exposure from early intrauterine life throughout childhood with respect to susceptibility of potential adverse effects.

1.1 MANGANESE

Manganese is an essential trace element with the atomic weight of 54.93. It can exist in eleven oxidation states ranging from -3 to +7, and the most common stable oxidation state is +2. In this state, Mn is soluble in water, and this is also the form most often found in the diet (WHO 2004; Martinez-Finley et al. 2013). Manganese metalloenzymes utilized in the metabolism include glutamine synthetase, arginase, phosphoenolpyruvate decarboxylase, and the antioxidant manganese superoxide dismutase (MnSOD) (Wood 2009). Manganese is needed for normal immune function, regulation of blood sugar and cellular energy, bone growth, and aids in the defense mechanisms against free radicals (Aschner et al. 2005). Manganese is present in various minerals and can thereby leach to surrounding ground water (WHO 2004). The industrial emission (such as ferroalloy production and iron and steel foundries) and emission from cars using gasoline with added Mn are the most important sources of airborne Mn. Air erosion of dust and soils are atmospheric sources of Mn, although airborne Mn concentrations without anthropogenic sources are low (0.01 to 0.07 µg/m3) (Lucas et al. 2015).

1.1.1 Human exposure

In general, food (especially cereals, rice, and vegetables) is the main source of human exposure. Elevated concentrations of Mn are also present in groundwater worldwide (Frisbie et al. 2012). Inhalation is an important route of Mn exposure in occupational settings (Levy et al. 2003). Children's exposure and effects of Mn differ from that of adults and depend on the stage of development, age, and exposure to other environmental elements and pollutants (ATSDR 2012). Although the Mn concentration in breast milk is low (3- 10µg/L) (Ljung et al. 2009), infant formulas may contain up to 400 µg/L (Ljung et al. 2011), and even more if

dissolved in contaminated drinking water. The recommended adequate intake (AI) for adults, including pregnant and lactating women, is 3 mg/day, and for young children it is 0.02–0.5 mg/day (EFSA 2013). However, AI for infants and children were extrapolated from the AI for adults. The adult AI was based on observed Mn intakes (based on dietary reference values) with a mixed diet (EFSA 2013).

1.1.2 Manganese kinetics

The amount of Mn absorbed across the gastrointestinal tract is variable, but typically averages about 3– 5%. Although the intake of Mn is higher from food than from the water, absorption from water may be higher as a result of higher bioavailability (ATSDR 2012). Absorption of Mn from oral ingestion can also be influenced by other factors, e.g. presence of other elements and compounds such as iron, calcium, phytate, ascorbic acid, and other constituents in the diets (Davidsson et al. 1991). Iron status may affect intestinal absorption of Mn. Previous studies indicated that absorption increased in individuals with iron deficiency anemia (Park et al. 2013), and decreased significantly in individual with adequate iron status (i.e. higher ferritin concentrations) (Finley 1999; Ljung et al. 2009). Usually, Mn and iron share common absorption and transport mechanisms, including protein transporters such as the divalent metal transporter-1 (DMT-1) or the transferrin (Tf)/Tf receptor (TfR) system (Fitsanakis et al. 2010; Park et al. 2013). Once Mn has been absorbed from the gut it is transported via the portal system and removed by the liver (Aschner et al. 2005). Manganese mainly concentrates in the tissues with high energy demand (brain) or that are rich in mitochondria, such as bone, liver, pancreas, and kidney (Aschner et al. 2005). The Mn concentration in the blood about 65% in the erythrocytes of healthy adults ranges from 4 to 15 µg/L (Milne et al. 1990; Ljung et al. 2009; ATSDR 2012). The main route of excretion for Mn is through the bile, with only a small proportion (about 1% of dietary intake) is eliminated via urine (EFSA 2013). The elimination time of Mn from the body varies, with a half-life between 13 and 37 days (Finley et al. 1994).

During pregnancy, the maternal blood Mn concentrations increase progressively (Spencer 1999; Takser et al. 2004), indicating increased intestinal absorption via the divalent metal transporter -1 (DMT1), the expression of which is up-regulated in conditions of low iron stores and in pregnancy (Kippler et al. 2009; Ljung et al. 2009). Adequate antioxidant defense is essential to protect the placenta from excessive oxidative stress, which may adversely affect fetal programming, as well as child growth and development (Thompson et al. 2012). In addition to the cytoplasmic copper and zinc superoxide dismutase, the mitochondrial

MnSOD functions as an antioxidant for detoxification of the superoxide anions in the placenta (Mistry et al. 2011). Manganese is actively transported across the placenta (Kippler et al. 2010), and at delivery, cord blood Mn concentrations are higher than the concentrations in maternal blood (Guan et al. 2013). Experimental studies have shown that the active transport is saturated at excessive Mn exposure, but simple diffusion of Mn across the placenta may still occur (Yoon et al. 2009), indicating that there may be a risk of excess fetal exposure. Several studies have shown that Mn absorption may be gender- and age-dependent (Finley et al. 1994; Erikson et al. 2007; Oulhote et al. 2014); neonates and infants absorb and retain a higher proportion of Mn than adults, and women absorb more Mn than men (Finley et al. 1994). During infancy, the regulation of the intestinal absorption of Mn and the biliary excretion are not yet fully functioning, (Davidsson et al. 1989; Aschner et al. 2005), which at high exposure may lead to greater risk of Mn toxicity.

1.1.3 Biomarkers of manganese exposure

There is yet no reliable biomarker of Mn to measure long-term exposure or body burden (Smith et al. 2007; Ljung et al. 2009). A reliable biomarker would facilitate preventive control of the Mn exposure for an individual, as well as population level. In epidemiological studies, Mn-levels in drinking water and in biological samples (i.e. blood, urine, hair, saliva, toenails, and deciduous teeth) have been used as biomarkers of exposure (Gunier et al. 2014; Andrade et al. 2015). The Mn concentrations in the blood (i.e., whole blood, erythrocyte, plasma, or serum) and in urine are used as biomarkers of exposure in occupational and population-based studies (Smith et al. 2007; Zheng et al. 2011). However, concentrations of Mn in blood are homeostatically regulated by the hepatic portal system, and Mn has a shorter half-life in the plasma (1-2 hours) than in the erythrocytes (in the order of months) (Lucaciu et al. 1997; Vitoux et al. 1999). The main limitations for the use of urinary Mn as a direct measure of exposure are high inter-individual variability in the urinary fraction, and only a small amount is actually excreted via urine (Gunier et al. 2014). Hair Mn has often been used to reflect environmental exposures (Eastman et al. 2013). Nevertheless, there are concerns in the use of hair Mn as a predictor of Mn body burden due to exogenous contamination, and the inter-individual variability in Mn concentrations due to differences in hair characteristics (Kempson et al. 2011). Recently, deciduous teeth (Arora et al. 2012; Gunier et al. 2013), saliva (Lucas et al. 2015) and toenails (Laohaudomchok et al. 2011) have been used to assess Mn exposure. However, limited evidence is available on the relationship between Mn levels in these tissues and exposure.

1.1.4 Health effects of manganese in adults

Manganese toxicity in humans via inhalation is well-recognized in occupationally exposed individuals (Sinczuk-Walczak et al. 2001; Roels et al. 2012). Manganese-induced neurotoxicity (manganism) following oral ingestion is of greatest concern and one of the most sensitive toxicological endpoints (Aschner et al. 2005). Manganism is associated with elevated brain levels of Mn, primarily in the areas known to contain high amounts of dopaminergic cells, such as basal ganglia and cerebral cortex (Guilarte 2013).

1.1.5 Health effects of manganese in children

The time between conception and birth is the most vulnerable period in life, during which the environment may have immediate and lasting effects on fetal growth and development (Selevan et al. 2000). The fetus undergoes rapid growth and organ development (**Figure 1**), which can be influenced by exposure to both essential and toxic elements such as Mn. Early childhood is also a critical period for the continued development and maturation of several biological systems such as the brain, lung, and immune system, and environmental toxicants can impair brain function and neurodevelopment also later in life (Selevan et al. 2000; Tarrade et al. 2015). When compared to adults, the developing brain in early-life might be more sensitive to excessive Mn exposures, due to the increased susceptibility during periods of rapid development (Grandjean et al. 2014). The development of single neurons is completed by 12 weeks of gestation, and the major part of the brain develop in the first two years following birth (Qiu et al. 2015).

There are epidemiological studies that have shown inverse associations of prenatal Mn exposure with fetal growth (Vigeh et al. 2008; Zota et al. 2009; Yu et al. 2013) and neuropsychological development in young children (Takser et al. 2003; Ericson et al. 2007; Lin et al. 2013; Yu et al. 2014). In addition, neurotoxicity related to excess Mn exposure has been shown experimentally, in neonates given parenteral nutrition, and in environmentally exposed children (Erikson et al. 2007). Moreover, recent studies have focused on associations of excessive Mn intake from drinking water with impaired child growth and neurodevelopment. One study evaluated Mn concentrations in water during pregnancy and fetal growth, and found that a combination of high water concentrations of Mn and iron was inversely associated with fetal growth (Grazuleviciene et al. 2009). A cross-sectional study in Bangladesh found that the odds ratio for infant mortality nearly doubled when drinking water Mn concentrations were greater than 400 µg/L (Hafeman et al. 2007). This finding was supported by an ecological study from USA, which showed a positive association between

water Mn and infant mortality (Spangler et al. 2009). Several studies have shown inverse associations of water Mn with cognitive and neurobehavioral performance in children (Wasserman et al. 2006; Bouchard et al. 2011; Khan et al. 2011). A recent review highlighted Mn neurotoxicity with respect to cognitive processes, timing of exposure, gender differences, susceptibility and exposure limits with regards to age (Roels et al. 2012). Although many of these studies point to inverse associations of Mn exposure in early-life with child health and development, most of them were cross sectional in design, had small sample sizes and did not follow up the children for a long period.
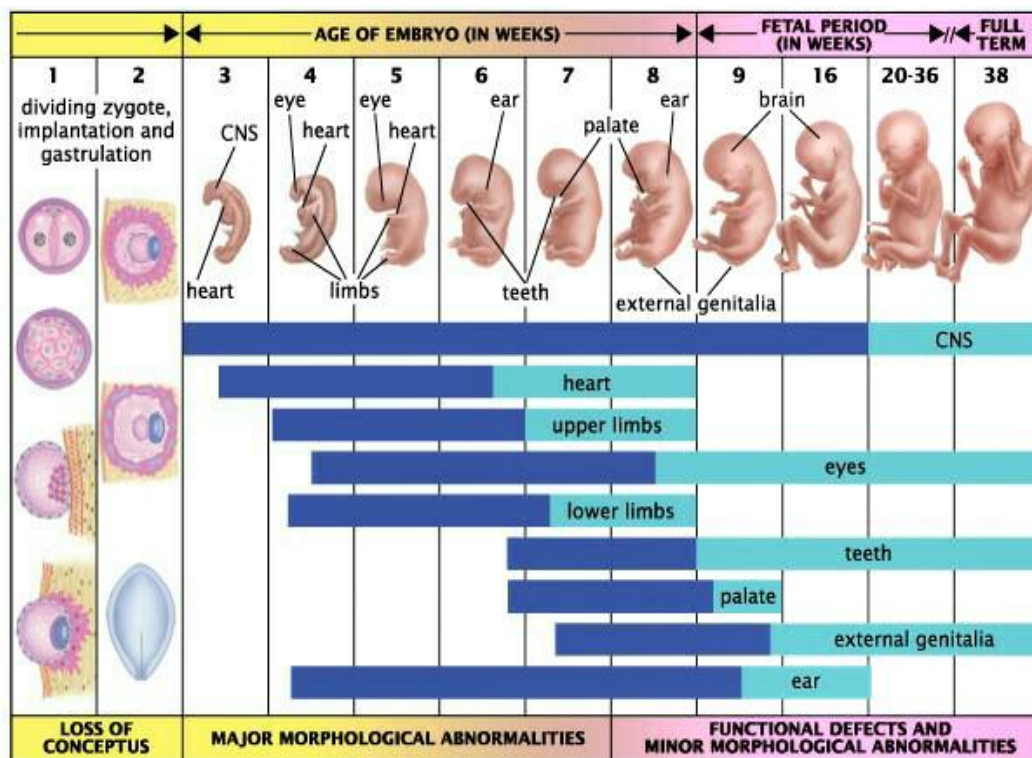

Figure 1. Schematic illustration of the sensitive or critical periods in fetal development. Blue bars denote highly sensitive periods, and light blue bars indicate periods at risk for minor abnormalities and functional defects. Modified and reprinted with permission of Prof. Beate Ritz, School of Public Health, UCLA (Ritz B 2008).

2 AIM OF THE THESIS

The most critical window of early-life manganese (Mn) exposure is not known. Thus, it is necessary to study potential effects of Mn exposure during the entire period from early intrauterine life to childhood.

The overall aim was to evaluate the potential effects of Mn on fetal and child health and development.

The specific aims were to elucidate

- the potential effects of exposure to elevated Mn concentrations via drinking water during pregnancy on fetal and neonatal survival **(Paper I)**, and on fetal growth **(Paper II)**,

- the potential effects of elevated Mn exposure via drinking water from fetal life to school-age on children's cognitive function and behavior at 10 years of age **(Paper III)**.

3 MATERIALS AND METHODS

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

3.1 STUDY AREA AND POPULATION

The prospective cohort studies in the present thesis were conducted in Matlab, a rural region of Bangladesh. In this area, the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) provides health services to about 110,000 inhabitants via a central hospital and four connected health centers. In addition, icddr,b has been implementing a population-based Health and Demographic Surveillance System (HDSS) since the 1960s (ICDDRB 2006), where community health workers visit households on a monthly basis to collect information on vital events such as pregnancies, births, deaths, and morbidities.

In the study area, a population-based food and micronutrient supplementation trial during pregnancy (Maternal and Infant Nutrition Interventions in Matlab; MINIMat) was implemented from November 2001 through October 2003 (Persson et al. 2012). Women reporting that their last menstrual period (LMP) was overdue were offered to take a urine pregnancy test. Those with positive tests were then referred to health clinics for confirmation of pregnancy by ultrasound (Doi et al. 2011). All confirmed pregnant women were invited to participate in the MINIMat trial if the following eligibility criteria were met: viable fetus by ultrasound examination, gestational weeks (GW) less than 14, and no severe illness. In total, the MINIMat study recruited 4,436 pregnant women in early pregnancy. Enrolled women were randomly assigned to either early (around GW9) or usual (GW20) start of food supplementation, and the two groups were again randomized into three micronutrient supplementation groups, resulting in a total of six groups. The micronutrient supplements consisted of either 30 or 60 mg iron and 400 µg folic acid, or a multiple micronutrient capsule with 15 micronutrients, including 30 mg iron and 400 µg folic acid (no manganese).

The present work is part of the ongoing studies on health effects of exposure to environmental pollutants, nested in the MINIMat trial (Vahter et al. 2006). In the study area, more than 90% of the families used drinking water from wells (ICDDRB 2007). It was revealed that 70% of the wells contained water with >10 µg/L of arsenic (WHO guideline value) (Rahman et al. 2006). The shallow wells (<50 m) with arsenic concentrations >50 µg/L (Bangladeshi guideline value) were painted red (dangerous), and those with arsenic concentrations <50 µg/L were painted green. Moreover, deep wells (>50 m) were dug more frequently, as this turned out to be the most efficient mitigation method to reduce exposure to

arsenic (Johnston et al. 2013; Hossain et al. 2014). Later on, it was found that over 40% of the wells contained >400 µg Mn/L (previous WHO guideline value) (Ljung et al. 2009).

All three studies **(Paper I-III; Figure 2)** in the present thesis were nested into the MINIMat trial. In total 3,971 pregnant women were identified between February 2002 to April 2003, and had information regarding birth outcomes and early fetal loss. These women constituted the cohort used in **Paper I,** exploring the association of Mn exposure in pregnancy with early fetal loss and birth outcomes. In **Paper II**, the pregnant women who had singleton births with measured birth anthropometry (n= 1,695) were included for analyses of associations of Mn exposure during pregnancy and size at birth. A subsample of the MINIMat cohort was selected for longitudinal follow-up of child growth and development (Hamadani et al. 2011; Kippler et al. 2012). In **Paper III**, we included the mothers and children (born between October 2002 and December 2003; n= 1,607) who were followed up for child growth and development at 10 years of age.
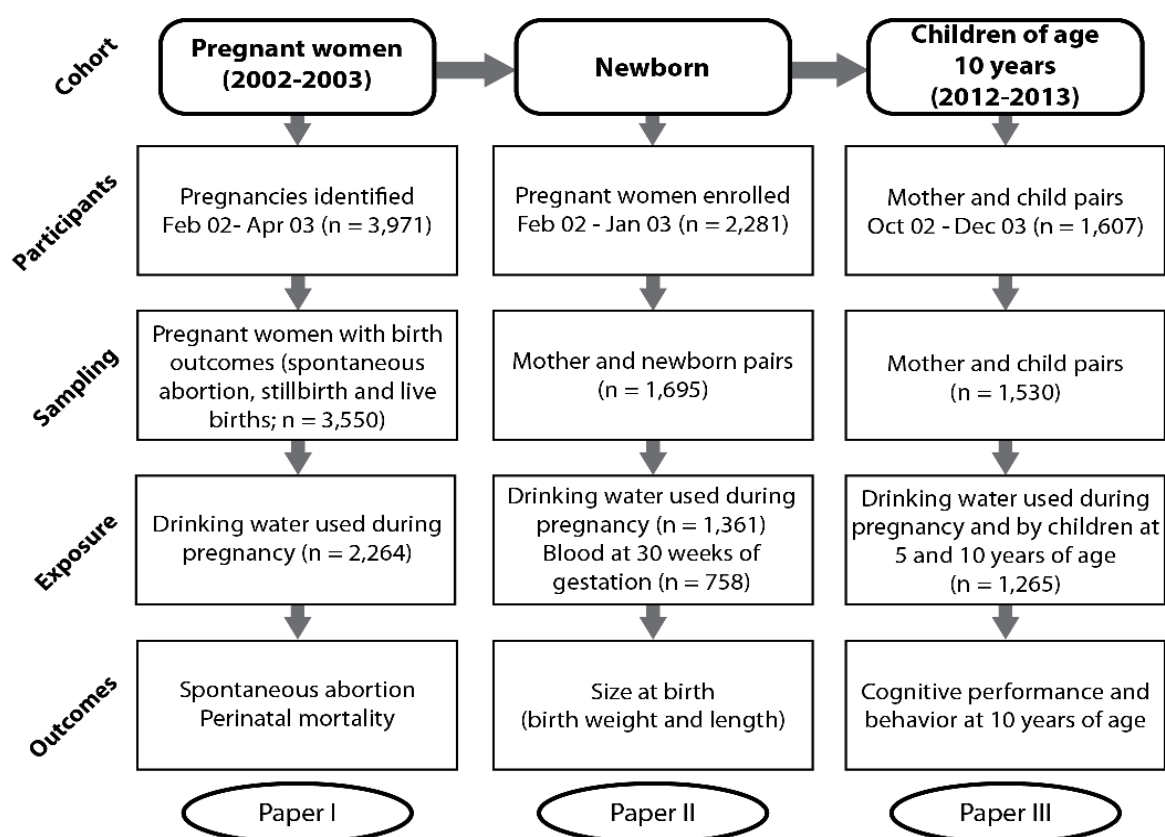

Figure 2. Flowchart depicting the study cohorts in the three different papers.

3.2 SAMPLING AND DATA COLLECTION

The present studies included drinking water and biological samples collected in different gestational weeks during pregnancy as well as in early childhood.

3.2.1 Drinking water samples

The women's drinking water used during pregnancy (**Paper I-III**) was available from a parallel population-based study (AsMat), which evaluated the potential health effects of arsenic in well water (Rahman et al. 2006). Information on lifetime drinking water sources were collected from all inhabitants of the study area via household interviews in 2002-2003. The children's drinking water samples (**Paper III**) were collected as a part of the current projects. We visited the families at the time of follow-up of the children at 5 and 10 years of age. The health workers interviewed family members about the water sources used by the children for more than three months, and collected water samples from all wells. The samples were stored at -20°C in Matlab for subsequent transport to Karolinska Institutet, Sweden, for analysis of Mn and other elements such as arsenic, iron etc. Aliquots of water samples were diluted 1:10 with 1% nitric acid, and analyzed for Mn and other elements using inductively coupled plasma mass spectrometry (ICPMS), as described in **Paper I-III**. No samples were below the limit of detection (LOD; three times the standard deviation of the blank) for water Mn (0.01 μg/L). Analytical performance was ascertained by analysis of a certified reference material (NIST 1643e, Trace Elements in Water, National Institute of Standards and Technology, Gaithersburg, MD), which showed good agreement with obtained results.

3.2.2 Biological samples (blood and urine)

Maternal venous blood samples were collected at 30 weeks of gestation from all pregnant women participating in the MINIMat trial. However, only a sub-sample of the women was available for measurement of Mn and other elements in the erythrocyte fraction, mainly due to blood being used for other purposes. The erythrocyte fractions were acid digested and analyzed using ICP-MS (**Paper II**). Samples of maternal urine were collected twice during pregnancy (GW8 and 30) and child urine at 5 and 10 years. Maternal (**Paper I-III**) and children's (**Paper III**) urinary concentrations of Mn, arsenic, cadmium, and lead were used as covariates.

3.2.3 Rice samples

Manganese and other elements were measured in samples of rice (**Paper I&II**) collected from 66 of the studied families. The rice samples were acid digested using a microwave

assisted high pressure digestion system and analyzed for metal concentrations by ICPMS as described in detail previously (Kippler et al. 2010). The fresh rice samples were rinsed with deionized water prior to digestion. To obtain dry weight, the rice samples were dried at 80°C for 12 hours. The mean decrease in weight after drying was 13% (Bergkvist et al. 2010).

3.3 COVARIATES

Detailed information on covariates has been described in the individual papers (**Paper I-III**). Information on the women's characteristics [age, early pregnancy weight and height, date of delivery, education, hemoglobin concentrations (Hb; g/L), early fetal loss and birth outcomes, and socioeconomic status (SES)], as well as the children's anthropometry at birth, breast feeding history, and infant morbidity or mortality were available from either the MINIMat trial or the HDSS database. Only two women reported smoking during pregnancy, and none used alcohol. During the follow-up of the children, we collected information on the number of siblings, years of education, and type of schools. The children's weight, height, and head circumference at 5 and 10 years of age were also measured.

3.3.1 Socioeconomic status

Socioeconomic status (SES) was based on a wealth index constructed from information on family ownership of a number of consumer items, housing structure, and dwelling characteristics using principle component analysis (Gwatkin et al. 2000; Saha et al. 2008). A weight was attached to each item from the first principal component. Categorical variables were transformed into separate dichotomous (0-1) indicators. Based on this wealth index, households were divided into SES quintiles: quintile 1 (poor), quintile 2 (lower middle), quintile 3 (middle), quintile 4 (upper middle), and quintile 5 (rich). The information was updated during the follow-up of the children at 10 years of age. The wealth index (range= -5 to +5) was used either as a continuous or categorical variable (SES quintiles) in **Paper I-III**. The wealth index has been used at country-level health and demographic surveys to measure inequalities in household characteristics, in the use of health and other services, and in health outcomes.

3.3.2 Pregnancy weight gain

In our study, we recorded the women's weight in early pregnancy (around GW9) and in late pregnancy (around GW30). Then, pregnancy weight gain **(Paper II)** was calculated by subtracting the weight measured at enrollment from the weight measured at GW30. Most previous studies have defined total weight gain as the difference between mother's weight at delivery or near delivery and pre-pregnancy weight [for review, see Lau et al (Lau et al.

2014)]. Most of those studies used self-reported pre-pregnancy weight or weight data subtracted from medical records. In this cohort, information on pre-pregnancy weight was not collected from the women and there were no medical records. The closest we could get to a pregnancy weight gain was to use the enrolment weight as a proxy for the pre-pregnancy weight.

3.4 OUTCOMES

The following section provides an overview and evaluation of different outcomes in the present thesis. A detailed description of a particular method can be found in the referenced papers (**Paper I-III**). Adverse pregnancy outcomes (spontaneous abortion and still birth) and infant mortality are common in the study area. Despite marked improvement in child survival during the last decade, still birth (35/1000 births), early neonatal mortality (34/1000 live births), and percentage of low birth weight babies (30%) are still high (Ronsmans et al. 2008; Persson et al. 2012). Arsenic has been associated with adverse pregnancy outcomes and increased risk of infant morbidity and mortality (Rahman et al. 2007; Rahman et al. 2010). In addition, it has been shown that exposure to both arsenic and cadmium (Cd; via food) are inversely associated with fetal growth (Rahman et al. 2009; Kippler et al. 2012) and lower intelligence scores in children in the present study area (Hamadani et al. 2011; Kippler et al. 2012). As the exposure to Mn in this population also turned out to be elevated (Ljung et al. 2009), it was essential to consider potential health effects of early-life Mn exposure.

3.4.1 Pregnancy outcomes and perinatal mortality (Paper I)

Spontaneous abortion (miscarriage) was defined as unintended loss of fetus before 28 weeks of gestation. We excluded all forms of induced abortion including "menstrual regulation" (vacuum aspiration within 10 weeks following a missed menstrual period). Perinatal mortality included both stillbirths and early neonatal deaths. Stillbirth was defined as the death of a fetus at 28 completed weeks of gestation or later. Early neonatal death was defined as the death of an infant within one week (seven completed days) of birth. According to current World Health Organization (WHO) recommendation, the perinatal period commences at 22 completed weeks of gestation and ends at seven completed days after birth. However, WHO recommends the definition of stillbirth or fetal death for international comparison as the death of a fetus commencing at 28 completed weeks of gestation and/or 1000g (WHO 2006). Information on the cause of neonatal death (<28 days) was based on a verbal autopsy questionnaire developed by the WHO and adapted for the Matlab HDSS area (Chowdhury et al. 2010).

3.4.2 Anthropometric measurements at birth (Paper II)

Among the pregnant women recruited to MINIMat, about 40% of the deliveries took place at health clinics, and weight and length of the babies were measured by the attending nurse (Persson et al. 2012). For women who delivered at home, a birth notification system was established to enable the measurements of birth anthropometry by trained health workers as soon as possible after birth. Birth weight and length were adjusted for those who were measured later than 24 hours after birth. Adjustment was done using a standard deviation (SD) score transformation, assuming that infants tend to remain in the same relative position in the anthropometric distribution during the first 24 hours, as described elsewhere (Arifeen et al. 2000; Persson et al. 2012).

3.4.3 Child development at 10 years of age (Paper III)

The children's cognitive function (IQ) was measured using the 4th version of Wechsler Intelligence Scale for Children (WISC-IV) (Wechsler 2003), and their behaviour was assessed using the Strengths and Difficulties Questionnaire (SDQ) (Goodman 2001), administered to the mothers (**Paper III**). In total, ten subtests (**Figure 3**) of the WISC-IV scores were used to generate four composite scores including *i)* verbal comprehension index (VCI), *ii)* perceptual reasoning index (PRI), *iii)* working memory index (WMI), and *iv)* processing speed index (PSI). The full scale IQ (FSIQ) scores were derived from the scaled scores (age adjusted scales of raw scores) of the 10 subtests. The SDQ consists of 25 questions concerning both positive and negative behavior of the children. There were five subscales, one for pro-social or social strengths (up to 10 points) and four for difficult or negative behavior (up to 40 points in total) (**Figure 3**).
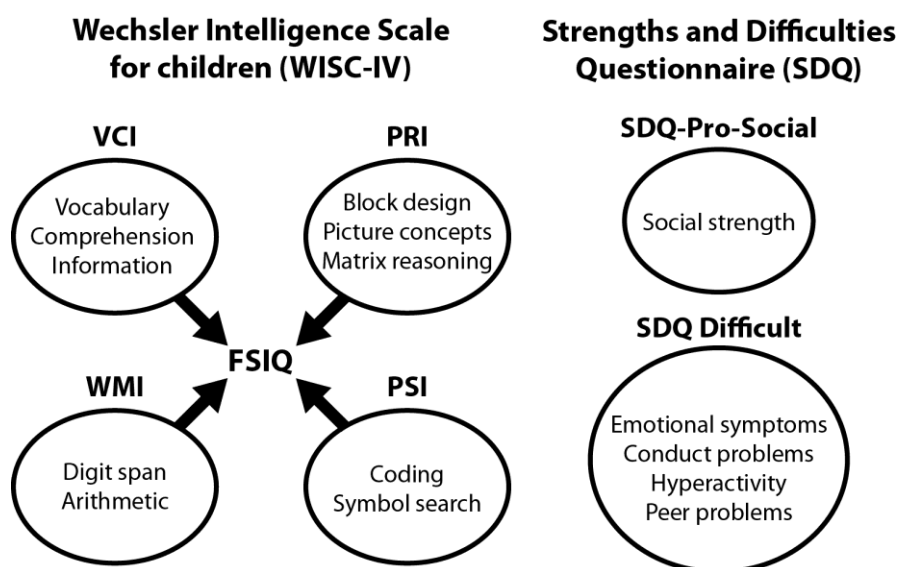

Figure 3. Assessment tools for children's cognitive function and behavior at 10 years of age.

3.5 ETHICAL CONSIDERATION

Written consent was obtained from the pregnant women before enrollment in the MINIMat trial. During developmental assessment of the children, written consent was obtained from the mother or legal guardian of each child prior to participation in the follow-up studies. Enrolled women 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 and Ethical Review Committees of icddr,b, Bangladesh and Karolinska Institutet, Sweden.

3.6 STATISTICAL ANALYSIS

Detailed descriptions of statistical analyses have been explained in **Paper I-III**. The analyses were conducted using SPSS (version 20.0/22.0, IBM Corporation, USA) and STATA (version 11; STATA Corp, College Station, TX, USA). Bivariate associations between continuous variables were assessed using Spearman's rank correlation coefficient (r_s). Mann Whitney U-test, Kruskal–Wallis non parametric test, or χ^2 test were used to test for difference between independent groups, whereas Friedman analysis of variance (ANOVA) was used to test for differences between multiple dependent groups. Associations of exposures with outcomes were initially examined by scatter plots (with moving fitted average line, Lowess), and further by regression analyses. If the Lowess lines indicated nonlinear associations, we used the Mn concentrations as a categorical variable in the regression analyses (i.e. tertiles; **Paper I-II**). We applied logistic regression with estimated odds ratios (ORs) and 95% confidence intervals (CIs) to explore the impact of Mn exposure on spontaneous abortion and perinatal death (**Paper I**). We applied linear regression analyses [B coefficient and 95% confidence intervals (CIs)] to assess the associations between Mn exposure on size at birth **(Paper II)** as well as IQ and behavior scores at 10 years (**Paper III**). We adjusted the regression models for variables that are known or proposed risk factors for all outcomes, or that were significantly associated with the exposure and outcome in the bivariate analyses (P <0.05). Missing data were handled by complete subject analysis. All tests were two-sided. Statistical significance was considered as a 95% CI that did not include zero (or 1 when calculating OR) or a P value <0.05.

4 RESULTS AND DISCUSSION

This section summarizes the main results of the present thesis along with a discussion regarding mechanisms and methodological considerations.

4.1 MANGANESE AND OTHER ELEMENTS IN WELL WATER

In Matlab, the median Mn concentration increased from about 200 µg/L (range=1.3- 6,550 µg/L) during pregnancy (**Papers I & II**) to 339 µg/L (range= 0.1 – 8,680 µg/L) when the children were 10 years old (**Paper III**), whereas arsenic concentrations decreased from 33 to 2.2 µg/L during the same period. This is probably because of the ongoing installation of deep wells, which contain higher levels of Mn and lower levels of arsenic (**Table 1**). Similarly, another study in this area evaluated well water Mn and arsenic based on aquifer's sediment color tools (a method recommended to local drillers for targeting low arsenic aquifers), and they found high Mn and low arsenic in red/off-white aquifer's present at a depth of 24-88 m (Hossain et al. 2014). The study group also observed that both Mn and arsenic concentrations were lower in wells deeper than 100 meter (Hossain et al. 2012), which is in agreement with our findings (**Table 1**). Moreover, we have found that both maternal and child water Mn concentrations were inversely correlated with the concentrations of arsenic in water (**Paper I &II; Table 1**).

Table 1.Concentrations of manganese and other elements in drinking water used by the mothers' during pregnancy and their children at 10 years of age by well depth.

Elements [a]	Mothers' water			Children's water		
	Well's depth in meter			Well's depth in meter		
	<50 (n= 412)	50-100 (n= 379)	>100 (n= 41)	<50 m (n= 335)	50-100 (n= 694)	>100 (n= 115)
Manganese (µg/L)	112 (27- 1,434)	937 (30- 3,186)	63 (62- 1,705)	98 (19- 2,686)	880 (30- 3,672)	162 (33- 1,885)
Arsenic (µg/L)	180 (0.83- 541)	2.4 (0.10- 112)	2.6 (0.26- 24)	114 (0.12-460)	1.3 (0.09- 152)	0.34 (0.03- 152)
Iron (mg/L)	3.6 (0.38- 11)	0.85 (0.07- 9.2)	2.0 (0.98- 11)	2.4 (0.14- 7.4)	1.2 (0.09- 7.8)	1.9 (0.13- 9.6)
Calcium (mg/L)	42 (12- 100)	41 (10- 93)	51 (14- 111)	37 (11- 85)	42 (14- 86)	44 (14-104)
Magnesium (mg/L)	20 (5.8- 41)	19 (6.6- 43)	22 (9.3- 51)	21 (6.1- 45)	25 (8.7- 50)	23 (6.4- 65)

[a] Data shown as median (5th -95th percentile)

In the fourth edition of the guidelines values for drinking water, the WHO discontinued the guideline value for Mn (<400µg/L) and concluded that the calculated health-based value was well above concentrations of manganese normally found in drinking-water, why it was not necessary to derive a formal guideline value (WHO 2011). Furthermore, review articles have indicated that the 400 µg/L health-based guideline was too high to adequately protect human

health (Ljung et al. 2007; Frisbie et al. 2012). In an attempt to evaluate the relative changes of water Mn and arsenic exposure over time, we stratified the cohort (all children) into four groups with different combinations of water Mn (below or above 400 µg/L) and arsenic concentrations (<10 µg/L or above) at 10 years of age (**Figure 4**). Only 19% of the children at 10 years of age and their families were exposed to both low Mn (<400 µg/L) and low arsenic (<10 µg/L). When we changed the cut-off for Mn concentrations to 1 mg/L (adverse effects mainly observed above this level in the present studies), we found that 30% of the families were exposed to low Mn (<1 mg/L) and arsenic (<10µg/L). In addition, about 40% of the children and their families were exposed to higher arsenic (>10 µg/L) or higher Mn (>1mg /L) concentrations.
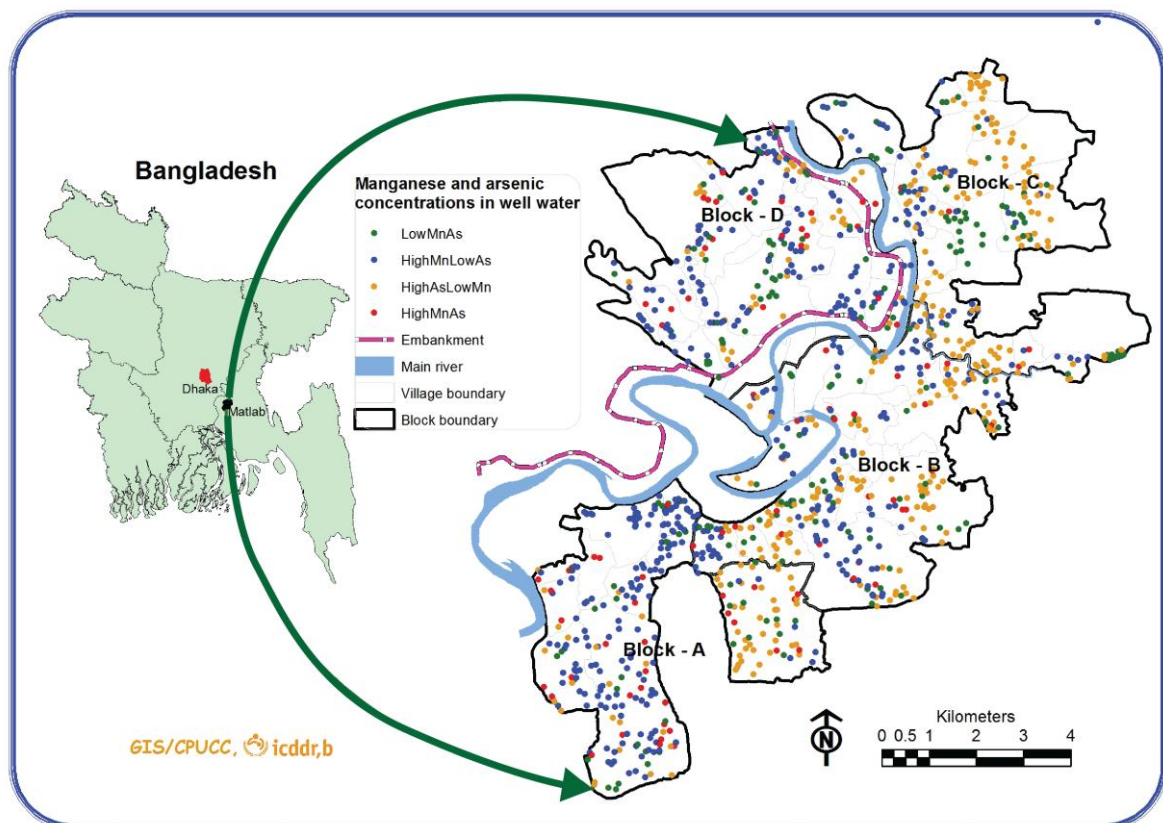
Figure 4. Contour map of manganese (Mn) and arsenic (As) concentrations (µg/L) in studied children's drinking water at 10 years of age. The four groups are Low Mn As (<400µgMn/L and <10 µgAs/L, shown in green), High Mn Low As ($\geq$400µgMn/L and <10 µgAs/L, blue), High As Low Mn (>10 µgAs/L &<400µgMn/L, yellow), and High Mn High As ($\geq$400µgMn/L and $\geq$10 µgAs/L, red)

In the studies within the present thesis, we adjusted our analyses for other elements (i.e iron, arsenic) in the drinking water which have previously been shown to be influential for pregnancy or early childhood outcomes (Rahman et al. 2007; Grazuleviciene et al. 2009;

Rahman et al. 2009). The median iron concentration was slightly higher in mothers' water (2.4 mg/L) compared to children's water at 10 years (1.5 mg/L) (**Table 1**). Possibly, the iron-rich water improved the iron status of the women, in accordance with reports from northern Bangladesh (Merrill et al. 2011). The water iron concentrations would contribute to the recommended dietary allowance (RDA) of 27 mg/day for pregnant women (Food and Nutrition Board 2001) by about 7 mg, assuming a daily intake of 3 L of water. However, 20% of the women in the present study cohort still had low iron stores (serum ferritin <15 µg/L) in early pregnancy (Li et al. 2008) and 33% were anemic (Hb<110 g/L) in late pregnancy (Persson et al. 2012). In a similar rural Bangladeshi population, the mean dietary calcium intake was estimated to be 350 mg/day (Hels et al. 2003), which is considerably lower than the RDA of 1,000 mg/day in pregnancy (Food and Nutrition Board 2001). Similarly, RDA of magnesium is 300 mg/day for adult women. In our study area, the concentrations of calcium and magnesium did not vary by the depth of the wells or time point. The water calcium (average 42 mg/L) and magnesium (average 22 mg/L) concentrations were similar to other Asian countries (calcium=2-80 mg/L and magnesium <20 mg/L) (WHO 2009). These concentrations in water may have made a small contribution to the daily requirements. In addition, deficiency in zinc, selenium, vitamin B12, and folic acid, is highly prevalent in this area (Lindstrom et al. 2011; Skröder et al. 2014).

4.2 MANGANESE BIOMARKERS

The identification and validation of exposure biomarkers is needed when assessing a dose–response relationship for the demonstration of cause and effect relationships (Smith et al. 2007; Andrade et al. 2015). For Mn exposure, studies have utilized a number of different biological markers such as whole blood, urine, hair, and teeth to demonstrate potential effects from exposures in occupational and environmental settings. In the present thesis (**Paper I-III**), we used the concentration of Mn in drinking water as the measure of exposure and Mn concentrations in erythrocytes (**Paper II**). Erythrocyte Mn (or blood Mn) is commonly used as a biomarker of exposure to Mn via inhalation or food. However, we found no correlation between maternal water Mn and erythrocyte Mn at 30 weeks of gestation (**Paper II**), not even for water Mn concentrations >1mg/L. The lack of correlation between Mn concentrations in drinking water and erythrocytes is in line with earlier findings (Wasserman et al. 2006; Mora et al. 2014). Most likely, the association may be influenced by the variable intake and uptake of Mn via water and food, and the strict homeostatic control of Mn in the body (Finley et al. 2003; Ljung et al. 2009). The children's drinking water Mn concentrations at 10 years was weakly correlated with urinary Mn levels ($r_s = 0.11$; $p= 0.001$), and indeed

only a small fraction is actually excreted via urine. Future research needs to identify reliable biomarkers of individual exposure to Mn via food and drinking water.

4.3   MANGANESE IN WATER AND PREGNANCY OUTCOMES (PAPER I)

The third of the pregnant women with highest water Mn exposure (median=1,292 µg/L) demonstrated approximately 35% lower incidence of spontaneous abortion than the third with the lowest exposure (median=56 µg/L) (**Figure 5).** Probably, this effect was related to the mitochondrial MnSOD in the placenta which protects against oxidative stress (Mistry et al. 2011). Furthermore, under normal conditions the level of plasma MnSOD rises towards the end of the first trimester (Jauniaux et al. 2000). Thus, Mn in drinking water might improve antioxidant capacity, particularly in undernourished women with high prevalence of zinc and copper deficiency, as in the present study population (Li et al. 2008; Kippler et al. 2009). We adjusted our analyses for other elements (such as arsenic and iron) in the water, which have previously been reported to be influential for pregnancy or early infant outcomes. Most importantly, we adjusted for arsenic exposure, which has been positively associated with spontaneous abortion and infant mortality in our study area (Rahman et al. 2010). Interestingly, the multivariable-adjusted regression model including both water Mn and arsenic (or urine arsenic) did not show any association of arsenic with spontaneous abortion (and arsenic did not change the estimate for Mn). Thus, in the previous analysis of arsenic exposure and spontaneous abortion, there might have been a protective effect of the elevated water Mn concentrations in the reference group as compared with that in the higher arsenic exposure groups (Mn and arsenic being inversely associated), causing biased OR elevation for spontaneous abortion in relation to arsenic exposure.
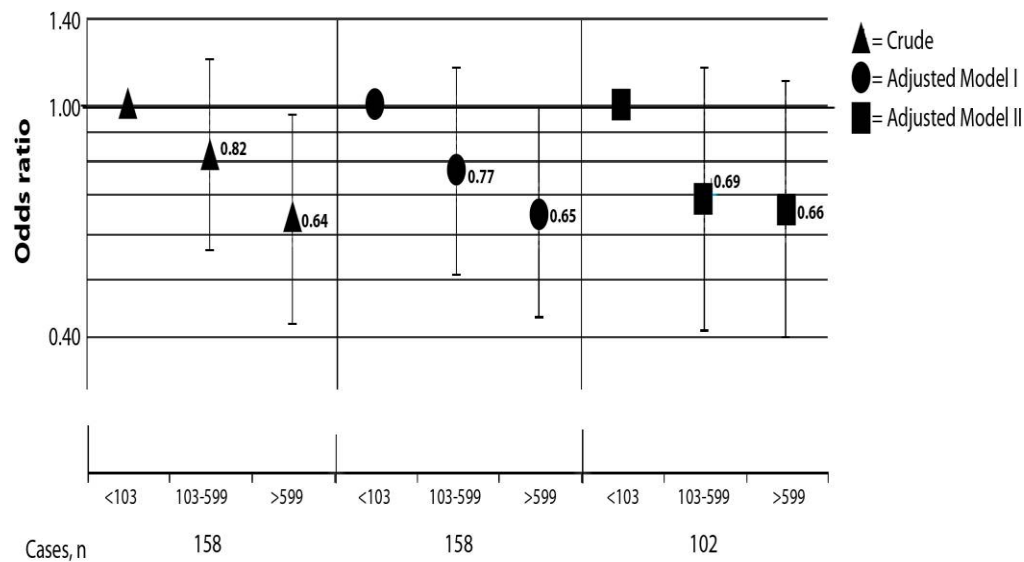
Figure 5. Association between water manganese (Mn) concentrations and spontaneous abortion. Model I has been adjusted for gravidity and concentrations of arsenic and iron in drinking water, and Model II was additionally adjusted for maternal education, BMI, and SES.

We did not find any indication of increasing perinatal mortality with increasing water Mn concentrations **(Figure 6).** Studies have suggested that perinatal deaths (i.e. stillbirth and early neonatal death) are largely the result of poor maternal nutrition, inadequate care during pregnancy, delivery, and the immediate post-partum period (Kusiako et al. 2000; Haws et al. 2009). In our study cohort, exposure to arsenic was not associated with still birth (Rahman et al. 2010). We could not directly compare our results with the only similar study (Hafeman et al. 2007), which reported a positive association between water Mn and infant mortality in Bangladesh, because they did not include still birth and did not define the time of infant death.
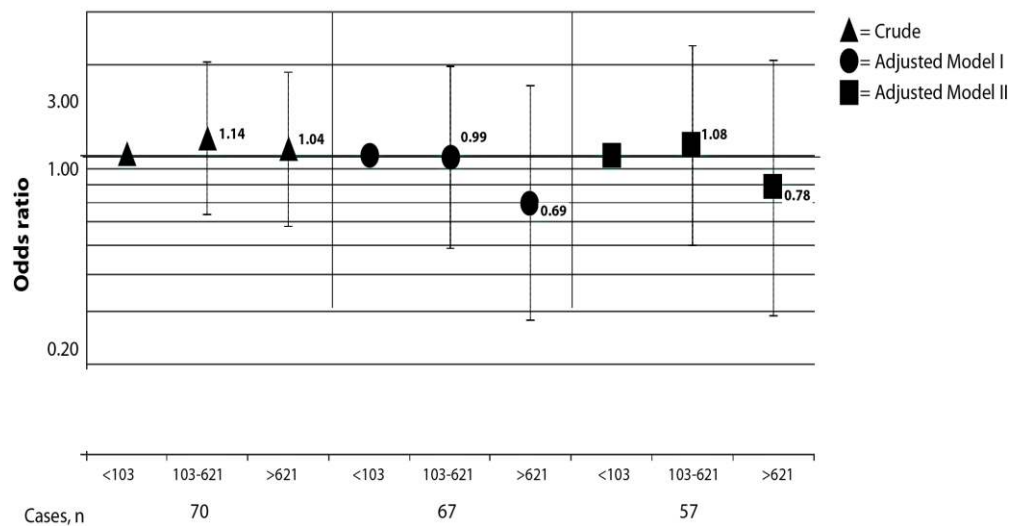
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Figure 6. Association between water manganese (Mn) concentrations and perinatal mortality. Model I was adjusted for gestational age at birth, maternal age, education, SES, season at birth and concentrations of arsenic, iron, magnesium, calcium, and zinc in water, and Model II was additionally adjusted for maternal BMI and place of delivery.

4.4 MANGANESE (IN WATER AND ERYTHROCYTE) AND FETAL GROWTH (PAPER II)

The newborns of mothers in the highest third of exposure to water Mn (median=1,495 µg/L) were on average 0.5 cm shorter (0.20 SD) than those in the least exposed third (median=56 µg/L; **Figure 7**). This difference was most pronounced in girls, but it was also apparent in boys born to mothers with low Hb values (<114 g/L), probably due to the more extensive absorption of Mn when the iron status is low (Kippler et al. 2009; Ljung et al. 2009; Wood 2009). Interestingly, the multivariable-adjusted regression model including both water Mn and either water or urinary arsenic did not show any association of arsenic with birth length, as observed in a previous study (Rahman et al. 2009). When we restricted the analyses to women with low water arsenic (<20 µg/L), this did not change the estimated effect of water Mn on birth length. In a subsample, we evaluated associations of erythrocyte Mn concentrations of the women during 30 weeks of gestation with birth weight and length.
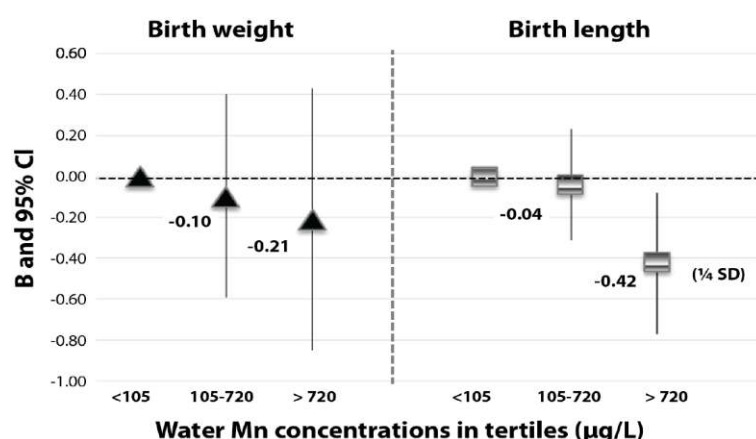
Figure 7. Associations of maternal water manganese concentrations with birth weight and length, adjusted for BMI, SES, parity, pregnancy weight gain, gestational age at birth, sex, water arsenic, and urinary cadmium. Birth weight is divided by 100 to fit the scale.

There was no correlation between the Mn concentrations in water and erythrocytes, and the association between erythrocyte Mn and birth length was less obvious (**Figure 8**). Possibly, women with high levels of Mn in drinking water experienced frequent peaks in plasma Mn (Michalke et al. 2007), leading to high fetal exposure and potentially impaired growth without any marked change in average erythrocyte Mn levels. The only previous study on water Mn and birth weight indicated an increased risk of low birth weight at term in relation to elevated water Mn and iron concentrations (Grazuleviciene et al. 2009). We did not observed an adverse effect of water Mn concentrations on birth weight; not even in women using water with high iron (>2.4 mg/L; median) concentrations.
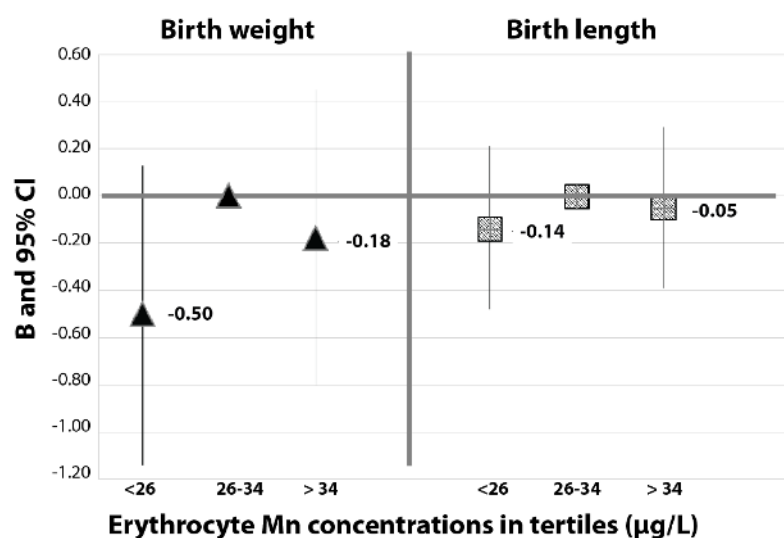

Figure 8. Associations of maternal erythrocyte manganese concentrations with birth weight and length, adjusted for BMI, SES, parity, pregnancy weight gain, gestational age at birth, sex, water arsenic, and erythrocyte cadmium. Birth weight is divided by 100 to fit the scale.

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We observed a tendency of an inverted U-shaped association between Mn concentrations in erythrocytes and birth weight (**Figure 8**). In fact, the erythrocyte Mn concentrations below about 30 mg Mn/kg were positively associated with birth weight, especially in the women with lowest BMI (< median; 19.7kg/m^2). This positive association may be related to the importance of Mn for multiple enzymes during fetal development (Erikson et al. 2007; Wood 2009; Mistry et al. 2011). However, we cannot exclude that the observed increase in birth weight with increasing erythrocyte Mn was, at least partly, due to improved iron status, which is known to affect birth weight (Balarajan et al. 2013). Moreover, we found a moderate correlation between erythrocyte Mn and iron up to about 34 µg Mn/kg (r_s= 0.29; *P*<0.001), i.e. the same concentration as where we found the change in the slope between erythrocyte Mn and birth weight**.**

4.5   MANGANESE IN WATER AND CHILD DEVELOPMENT (PAPER III)

The Mn levels in maternal drinking water were positively associated with their children's difficult behaviour at 10 years of age. For each increase of one mg Mn/L (median =0. 34 mg/L), these scores increased by about 0.5 points (0.13 SD) for the boys and 0.8 points (0.21 SD) for the girls born to anemic mothers (Hb<110 g/L), probably due to their more extensive absorption of Mn. The maternal water Mn concentrations (<3mg/L) and children's water at 10 years were positively (<0.15 SD) associated with the pro-social behavior scores in girls but not in boys. The positive association with water Mn concentrations at 10 years of age was similar to that found with the maternal water Mn. We did not find any similar finding in the literature, hence, it may be a mere chance finding and not of much public health importance. The effect-estimates for the association of water Mn with both behavioral scores did not differ in children of the women with low or high water arsenic concentrations.

Early-life Mn exposure seemed to affect the children's IQ, but we did not observe any clear evidence of adverse effects of water Mn in the cross-sectional analyses at 10 years of age. The associations between water Mn concentrations (especially at 5 years of age) and the boys´ cognitive function was generally inverse**.** For the girls' cognitive function, there were mainly positive associations with Mn levels in maternal drinking water up to about 3 mg/L, but not at higher concentrations (>3 mg/L). However, inverse associations of maternal water Mn concentrations with IQ scores were pronounced in boys (**Figure 9**) and somewhat evident in girls born to anemic mothers (**Figure 10**). Manganese is thought to primarily disturb the dopaminergic neuronal system, and experimental studies indicate increased susceptibility to excess Mn early in life (Tran et al. 2002; Beaudin et al. 2013). This was confirmed by our

findings and was especially pronounced in the children of anemic mothers, who likely absorb more Mn (Kippler et al. 2009; Ljung et al. 2009; Meltzer et al. 2010). Our results also indicate that the association between water Mn and IQ scores was influenced by the water arsenic concentrations, as restricting the analyses to the children of women with water arsenic concentrations (<20 µg/L) decreased the effect estimates for Mn by about 30 percent.
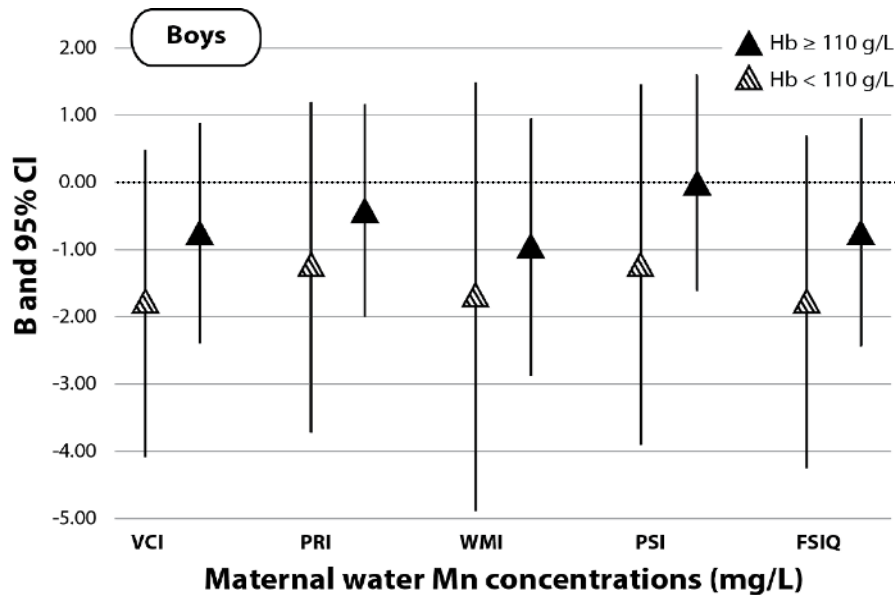

Figure 9. Associations of maternal water manganese concentrations with IQ scores in the boys of the women with (Hb<110g/L; n=103) or without anemia (Hb≥110 g/L; n=152), adjusted for mother's IQ, SES, children's (10 years) education, HAZ, Hb concentrations, types of school attended, HOME, tester, number of siblings, and mother's urine arsenic at gestational week 8.
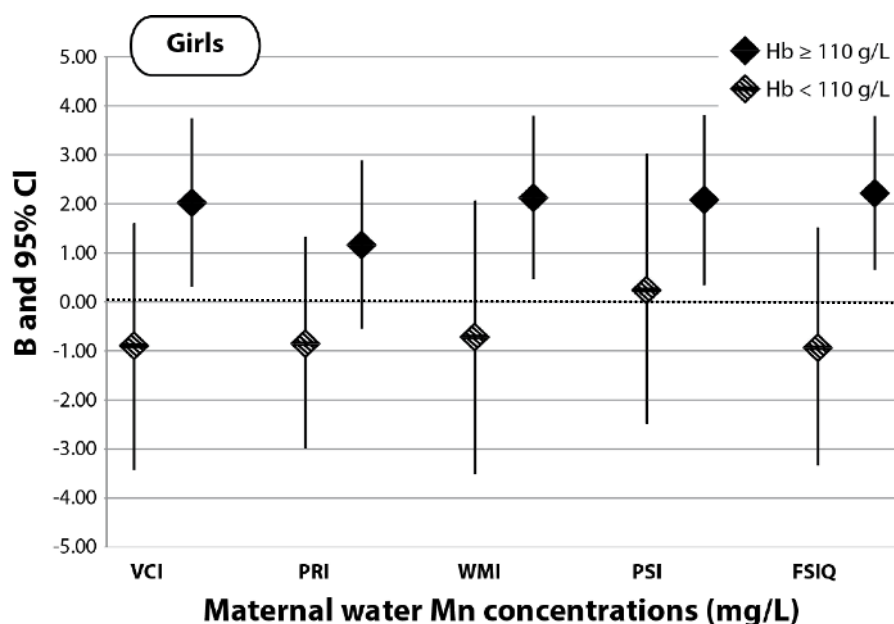
Figure 10. Associations of maternal water manganese concentrations (<3 mg/L) with IQ scores in the girls of the women with (Hb<110g/L; n=81) or without anemia (Hb≥110 g/L; n=134). Adjusted for mother's IQ, SES, children's (10 years) education, HAZ, Hb concentrations, types of school attended, HOME, tester, number of siblings, and mother's urine arsenic at gestational week 8.

4.6 HEALTH RISK ASSESSMENT

Usually, the main source of Mn is food, such as rice, the staple food in Bangladesh. A daily consumption by a Bangladeshi woman is approximately 300 g rice (dry weight) and 3 liters at water (Watanabe et al. 2004; Ohno et al. 2007). We measured Mn concentrations in samples of rice collected from 66 of the studied families, and found on average 7 mg Mn/kg (dry weight; inter quartile range 4.1- 9.9 mg Mn/kg), which would provide about 2 mg Mn/day from rice **(Paper I-II)**. In addition, women in the highest tertile of Mn exposure (median=1.3 mg/L; n= 620; **Paper I** and median=1.5 mg/L; n= 360; **Paper II**) received almost 4 mgMn/day from water. The adequate daily intake has been estimated to 3 mg during pregnancy (EFSA 2013), which means that about 30 % of the women exceeded their AI by 2-fold.

Rice would also be an important source of Mn for the young children in this area, as over 90% of the children living in Matlab were given solid foods, mainly rice products, in addition to breast milk at one year of age, after which solid food intake increases continuously (Saha et al. 2008). The elevated concentrations of Mn in drinking water will also contribute by about 0.3 mg to the daily intake, based on the assumption that the children consume around

1L of water per day (Gardner et al. 2011). Therefore, more than half of the children would receive the adequate amount of Mn (0.2 – 0.5 mg/day) from drinking water only. However, the contribution from water likely varied considerably between individuals and by seasons (at season with high temperature, individuals tend to drink more water). Considering the likely higher bioavailability of water Mn compared with Mn in the phytate-rich rice-based staple food (Davidsson et al. 1995), the Mn-rich water obviously provided much more Mn than needed.

4.7 GENDER DIFFERENCES

We reported sex differences in **Paper II & III**. We observed that for fetal growth, girls were more susceptible than boys, however, for neurodevelopment, boys were more susceptible to elevated water Mn concentrations in early-life. Supporting our observations, there are several lines of evidence from both human and experimental studies where the response to environmental exposure is different in boys and girls. To note, sex-specific changes in the epigenome are generated after fertilization, before adrenal and gonad differentiation and production of sex hormones (Gabory et al. 2012), which is a critical period for environmental influences (Barker et al. 2013). Indeed, previous studies from this cohort showed that maternal exposure to arsenic and cadmium in early pregnancy was associated with sex-specific alterations in DNA methylation in the newborns (Kippler et al. 2013; Broberg et al. 2014). Thus, sex-differences in the epigenome may explain the different results between boys and girls that we find in **Paper II & III**.

It has been suggested that female babies may be at lower risk of developing metabolic and neurodevelopmental disorders than male babies due to the presence of fetal estrogen (Roy et al. 2015) **(Paper III)**. The estrogen hormone, which is higher in girls than boys, may play a protective role in the regulation of neuronal structure and brain function (Vahter et al. 2007). This hormone may, through activator protein (AP)-1 and mitogen activated protein kinase (MAPK) pathways, regulate the MnSOD activity and reduce brain endothelial mitochondrial reactive oxygen species (ROS) production (Razmara et al. 2008; Than et al. 2009).

In addition, there is increasing evidence for sex-specific relationships between various environmental influences on the placental function and the risk of disease later in life (Gabory et al. 2013; Davis et al. 2014; Tarrade et al. 2015). Thus, many forms of developmental delay may originate in placental insufficiency (Walker et al. 2015). Hypoxia (deficient oxygen, O_2 supply) and oxidative stress are two disorders which occur in placental insufficiency (Herrera et al. 2014). Adequate antioxidant defense is essential to protect the placenta from excessive oxidative stress. It can be speculated that the observed apparent positive effects of the

prenatal Mn exposure (<3 mg/L) on IQ scores and pro-social behavior in girls (**Paper III**) might be related to the importance of mitochondrial MnSOD as an antioxidant in the placenta (Mistry et al. 2011). However, the specific mechanisms of the observed gender differences remain to be elucidated.

4.8 ADDITIONAL METHODOLOGICAL CONSIDERATION

The strengths of all three studies include the population-based prospective design, the large sample size and information on the women's drinking water during pregnancy (**Paper I-III**) and their children's water Mn concentrations at multiple time points (**Paper III**). Moreover, there was a wide range of Mn concentrations in the drinking water, as measured by a reliable ICP-MS method (**Paper I-III**).

In addition, in this thesis we were able to control for several potential confounders. Confounders may also be misclassified and several strategies were undertaken to decrease the level of misclassification. For instance, the inverse association between water Mn and arsenic showed a complex pattern. There may have been residual confounding, even after adjustment for arsenic in urine, a biomarker of ongoing arsenic exposure (**Paper I-III**). Therefore, we also tried to adjust for water arsenic instead of urine arsenic (categories or natural log transformed) in the models (**Paper I-III**). Finally, we restricted the models to women or their children with very low water arsenic concentrations. In **Paper III**, to minimize potential performance bias, we adjusted for tester in the regression models when exploring associations of water Mn and IQ and behavior scores. Although, we cannot exclude that there may have been residual confounding even after adjustment for tester.

In all three studies the major limitation was a lack of water samples or reliable information concerning the well water used by the women during pregnancy. To minimize the exposure misclassification, we evaluated reliability of water samples based on the agreement between the concentrations of arsenic in the water and arsenic metabolites in the urine (Vahter et al. 2006). In **Paper I-III**, we adjusted for socio-demographic characteristics and outcome differences between subjects for whom we had water samples and those without, limiting potential influence on the findings. To further minimize exposure misclassification, all (water and blood) analyses were performed with a high analytical accuracy. We performed several sensitivity analyses with regard to water Mn in order to test the robustness of the results. There is always a risk that the results are dependent on how the exposure categories are chosen. In **Paper I-II**, several attempts were made to explore possible effects of the categorization. The exposure (water Mn) was included either in tertiles or as a continuous variable (mg/L or natural log transformed).

We evaluated interactions between iron and Mn by Hb status of the women during pregnancy. However, Hb is not specific for iron deficiency. The serum ferritin and soluble transferrin receptor are considered reliable markers for iron status (Akesson et al. 1998). Availability of reliable makers of iron status for the studied women and their children would have helped our evaluation of interactions between Mn and iron status. In a previous study of a sub-sample from this cohort, an inverse association was observed between erythrocyte Mn and plasma ferritin concentrations at gestational week 14, supporting an increased Mn absorption at low iron status (Ljung et al. 2009). Unfortunately, we did not even have Hb concentrations of the children at 5 years. Therefore, we did not evaluate a potential interaction with Hb status at 5 years for the associations between children's water Mn at 5 years and IQ at 10 years. However, at 10 years of age, only 18% were anemic, leading to a lower uptake of Mn and possibly lower toxicity from excess drinking water Mn.

5 GENERAL DISCUSSION

The studies were conducted in Matlab, a rural part of Bangladesh, and included pregnant women and their children with generally low socioeconomic conditions, high prevalence of nutritional deficiency, and a wide use of Mn and arsenic contaminated groundwater for drinking purposes. The period from pregnancy to early childhood is considered as the critical window of vulnerability to environmental pollutants and toxicants (Walker et al. 2007). Exposure to Mn during this period exerts adverse effects on the growth and development, as shown by the studies presented in this thesis.

Growth retardation and nutritional deficiencies, mainly iron and iodine deficiency, are important predictors for poor neurodevelopment of children (Walker et al. 2007; Rydbeck et al. 2014; Skröder et al. 2014). The iron deficiency during pregnancy and childhood can enhance the absorption of Mn, probably because of a shared transport mechanism in the gut (Ljung et al. 2009; McArdle et al. 2014). In **Paper II & III**, we observed that toxic effects of Mn were more pronounced in children of anemic mothers. Thus, preventive measures concerning nutrition, in particular intake of iron, should be promoted, both for the benefits of adequate iron intake in itself, but also to avoid an increased absorption of Mn.

Arsenic in drinking water is often highly elevated in many rural areas in Bangladesh (Vahter et al. 2006; Chakraborti et al. 2010). Currently, the most efficient mitigation method seems to be construction of deep wells (Johnston et al. 2013), and the use of these wells has been encouraged to prevent the people from being exposed to arsenic (Hossain et al. 2014).

Additional mitigation options included installation of pond sand filters, Safi filter, home-based "3-pitcher" filters, and rainwater harvesters (Jakariya et al. 2007; von Bromssen et al. 2007). Since elevated Mn concentrations in drinking water are common around the world, the results obtained in the present thesis may have major importance for public health. However, the benefit of the low concentrations of arsenic in drinking water is most likely higher than the observed modest Mn-related effect on fetal growth and cognitive and behavioral development. To reduce arsenic and Mn in drinking water several measures could be taken, such as *i)* screening for both arsenic and Mn by using sediment color tools (Hossain et al. 2014) during installation of new wells *ii)* installation of deeper wells (>100 meter), which may provide dual benefits to vulnerable populations like our study area, by reducing not only both arsenic and Mn, but also the risk of childhood diarrheal disease (Winston et al. 2013), *iii)* treatment of water by oxidizing filters coated with Mn oxide to improve the performance of sand filters already in use (Jessen et al. 2005; Piispanen et al. 2010).

6 CONCLUSIONS

In summary, we may draw the following conclusions from this thesis.

- The Mn concentrations in drinking water varied widely, with higher concentrations of Mn in deep wells.

- Elevated concentrations during pregnancy appeared to prevent early fetal loss, an effect possibly related to the role of Mn in placental antioxidant defenses in early pregnancy.

- Elevated water Mn was associated with lower birth length, and newborn girls appeared to be were more susceptible than boys.

- The associations between erythrocyte Mn concentrations and size at birth were less evident.

- Exposure to elevated water Mn of the mother throughout pregnancy and children's early life (at 5 years of age) appeared to affect child neurodevelopment at 10 years of age adversely, especially when the mother was anemic during pregnancy. The boys were more susceptible than girls, for both cognitive function and difficult behavior.

As Mn absorption tends to be higher in anemic women, the existing interventions in routine program need to be strengthened. This includes routine assessment of anemia during pregnancy and supplementation of iron and folic acid during pregnancy and the postnatal period. Considering higher absorption of Mn in case of anemia in early life, screening children for anemia and treating them would be an important future public health intervention. During installation of new wells screening for both Mn and arsenic by using sediment color tools and digging into an aquifer that is low in both arsenic and Mn (deeper than 100 m) should also be promoted.

7 FUTURE RESEARCH

In my opinion, future research in this area should focus on:

- Prospective follow-up of the children studied here to explore the potential health effects of long-term manganese exposure later in life.
- Identification of reliable biomarkers of individual exposure to manganese via food and drinking water.
- Mechanisms involved in the early-life toxicity of manganese.
- Epidemiological studies in other populations designed to confirm the gender differences observed here.

In general, future research should focus on the potential adverse effects of manganese rich water on child growth and development to ensure long-term health.

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