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INTERACTIONS BETWEEN CADMIUM AND MICRONUTRIENTS IN PREGNANT AND LACTATING WOMEN

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To my family

ABSTRACT

The heavy metal cadmium is a widely dispersed environmental pollutant that has no biological function in the human body and is known to cause several adverse health effects, mainly on kidneys and bone and the endocrine system. Little is known about effects in early life. The main source of exposure in a non-smoking population is via basic foods such as cereals (e.g. wheat and rice) and vegetables. In adults, the gastrointestinal absorption of cadmium is generally low (<5%), but appears to vary depending on the type of diet consumed and the nutritional status of the individual. Increased absorption of cadmium at low iron stores has been documented both in experimental- and epidemiological studies, while interactions with other essential micronutrients (e.g. zinc and calcium) have been reported in experimental studies only.

The overall aim of the present thesis was to assess the maternal and early life exposure to cadmium with particular focus on the interactions with different essential micronutrients in pregnant and lactating women. The studies were nested into a large food and micronutrient supplementation trail conducted on pregnant women in a rural area of Bangladesh called Matlab. Most of the women in this area are malnourished and their main staple food is rice, known to easily take up cadmium from soil, suggesting that these women are at risk of increased uptake and accumulation of cadmium.

Maternal cadmium exposure was assessed via measurements of concentrations in maternal urine and blood (erythrocyte fraction), while the early life exposure was assessed via measurements in umbilical cord blood and breast milk. Besides cadmium, several nutritional markers were measured in blood, placenta and breast milk. Most of the measurements were performed using inductively coupled plasma mass spectrometry (ICPMS) after microwave assisted high temperature acid digestion (all samples but urine).

The results from the present studies show that women (aged 14 to 44 years) in rural Bangladesh have elevated cadmium exposure. In fact, the urinary cadmium concentrations were similar to those associated with increased risk of adverse health effects on kidneys and bone in other countries. As none of the Bangladeshi women were smokers the main source of exposure is likely food, probably rice.

Women with low iron stores had significantly higher uptake and accumulation of cadmium, indicating increased intestinal uptake of cadmium via the intestinal iron transporters divalent metal transporter 1 (DMT1) and ferroportin 1 (FPN1). Also, the intestinal uptake of cadmium was significantly increased during pregnancy, especially in women with low iron stores. A new finding in the present study is the strong positive association between cadmium and manganese in blood. This is most likely explained by the fact that manganese, like iron and cadmium, is transported via DMT1, leading to a parallel intestinal uptake of these three metals via DMT1. There was no evidence of cadmium uptake via zinc transporters. However, the increased intestinal uptake and accumulation of cadmium at low iron stores was more pronounced among women with adequate zinc status, indicating that zinc is important for the regulation of DMT1. In addition, cadmium appeared to inhibit the transport of calcium to blood.

The cadmium concentration in umbilical cord blood was low, but increased with increasing maternal exposure. There was an accumulation of cadmium in the placenta, which resulted in impaired zinc transfer to the developing offspring. The potential developmental consequences of this need further investigation. The mechanisms behind the interaction between cadmium and zinc in placenta are not known, but the present study indicated that it might be mediated via other mechanisms than the previously proposed binding to metallothionein (MT).

The transfer of cadmium to breast milk was low, but seemed to be mediated via the active transport system utilized by iron and manganese. In addition, cadmium appeared to impair calcium transport in the mammary gland, like in the intestine, possibly by blocking calcium channels and/or by binding to calcium transporters.

Keywords: Cadmium, Iron, Manganese, Calcium, Zinc, Micronutrients, Pregnancy, Breast milk, Placenta, Metallothionein

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals

I. **Kippler M**, Ekström E-C, Lönnerdal B, Goessler W, Åkesson A, El Arifeen S, Persson L-Å, Vahter M (2007). Influence of iron and zinc status on cadmium accumulation in Bangladeshi women. *Toxicology and Applied Pharmacology* 222: 221-226

II. **Kippler M**, Goessler W, Nermell B, Ekström E-C, Lönnerdal B, El Arifeen S, Vahter M (2009). Factors influencing intestinal cadmium uptake in pregnant Bangladeshi women – A prospective cohort study. *Submitted*

III. **Kippler M**, Lönnerdal B, Goessler W, Ekström E-C, El Arifeen S, Vahter M (2009). Cadmium interacts with the transport of essential micronutrients in the mammary gland – A study in rural Bangladeshi women. *Toxicology* 257: 64-69

IV. **Kippler M**, Hoque AMW, Raqib R, Öhrvik H, Ekström E-C, Vahter M (2009). Accumulation of cadmium in human placenta interacts with the transport of micronutrients to the fetus. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATSDR	Agency of toxic substances and disease registry
BMI	Body mass index
CaATPase	Calcium adenosine triphosphatase
CaT1	Calcium transporter 1
DMT1	Divalent metal transporter 1
EC	European commission
EFSA	European food safety authority
Ery-Cd	Erythrocyte cadmium
FPN1	Ferroportin 1
GFR	Glomerular filtration rate
HDSS	Health and demographic surveillance system
HRP	Horse-radish-peroxidase
IARC	International agency for research on cancer
ICDDR,B	International centre for diarrhoeal disease research, Bangladesh
ICPMS	Inductively coupled plasma mass spectrometry
JECFA	Joint FAO/WHO expert committee on food additives
LOD	Limit of detection
MINIMat	Maternal and infant nutrition interventions of Matlab
MRE	Metal responsive elements
MTF-1	Metal-responsive transcription factor-1
MT	Metallothionein
NAG	N-acetyl-β-glucosaminidase
p-Ft	Plasma ferritin
PTWI	Provisional tolerable weekly intake
p-Zn	Plasma zinc
RBP	Retinol binding protein
RDA	Recommended dietary allowance
Tf	Transferrin
TfR	Transferrin receptor
TWI	Tolerable weekly intake
U-Cd	Urinary cadmium
UNEP	United nations environment programme
WHO	World health organization
ZIP4	Zrt-like, Irt-like protein 4
ZIP8	Zrt-like, Irt-like protein 8
ZIP14	Zrt-like, Irt-like protein 14
ZnT1	Zinc transporter 1
ZnT5	Zinc transporter 5

1 INTRODUCTION

The present thesis includes studies on the maternal and early life exposure to cadmium as well as the interactions between cadmium and different essential micronutrients in pregnant and lactating women. Cadmium is known to cause several adverse health effects in humans, mainly on kidneys and bone. The main sources of cadmium exposure are food and smoking (WHO, 1992). In general, women have higher cadmium body burden than men (Nishijo *et al.*, 2004; Vahter *et al.*, 2007), which seems to be related to gender differences in nutritional status. Probably, early life is a critical window of exposure to many environmental pollutants, including cadmium. Thus, it is particularly important to monitor the cadmium exposure in pregnant women and their offspring, as well as to study factors that may influence the exposure, uptake and accumulation of cadmium.

1.1 CHEMISTRY AND USE OF CADMIUM

Cadmium is a toxic metal that belongs to group IIb in the Periodic table. The naturally occurring isotopes of cadmium are 106 (1.22%), 108 (0.88%), 110 (12.39%), 111 (12.75%), 112 (24.07%), 113 (12.26%), 114 (28.86%), and 116 (7.50%) (WHO, 1992). Cadmium is widely but sparsely distributed in the earth's crust, primarily as sulfide minerals in association with zinc ores, and, to a lesser degree, lead and copper ores (ATSDR, 2008). The pure cadmium metal is soft and has a silver-white appearance. Cadmium was first discovered in 1817 as an impurity in zinc carbonate, but it was not until the first part of the 20th century that cadmium came into wide industrial use. It has been extensively used as a color pigment (yellow, orange, and red), a stabilizer for plastics (e.g. in polyvinyl chloride), in coatings and plating's, and as a cathode in batteries (nickel-cadmium batteries), among other uses. In 2005, nickel-cadmium batteries accounted for about 82% of the estimated world consumption (UNEP, 2006). It should be noted, that during the period from 1995 to 2005 the primary production of cadmium has increased steeply in Asia (e.g. China, Japan, Korea) and decreased considerably in Europe (UNEP, 2006).

1.2 CADMIUM IN THE ENVIRONMENT

Cadmium is emitted to air, soil, and water via non-ferrous metal mining and refining, manufacturing and application of phosphate fertilizers, application of sewage sludge and farmyard manure, fossil fuel combustion, and waste incineration and disposal. In air, most cadmium is bound to small-size particles (<1 μm) or vapor (EC, 2001). It is

emitted to the atmosphere as elemental cadmium and/or as cadmium oxide, cadmium chloride, or cadmium sulphate (EC, 2001). In the atmosphere, cadmium can be transported many kilometers from the emission. The atmospheric residence time is about 1 to 10 days before deposit onto soil and/or water surfaces via wet or dry processes (Elinder *et al.*, 1985; ATSDR, 2008). Increased levels of cadmium in agricultural soils lead to increased levels in food and animal feed since cadmium is easily taken up by plants (UNEP, 2006). The mobility of cadmium in soil is dependent on several factors such as pH, presence of organic and inorganic ligands, and competition from other metal ions (OECD, 1994). In water, cadmium can exist as a free ion or as complexes with other inorganic or organic substances (EFSA, 2009). Both cadmium sulphate and cadmium chloride are quite soluble in water, whereas elemental cadmium, cadmium oxide, and cadmium sulphide are almost insoluble (EC, 2001). The mobility of cadmium in water is enhanced by low pH, low hardness, low levels of suspended matter, high redox potential, and low salinity (UNEP, 2006).

1.3 EXPOSURE TO CADMIUM

In the general non-smoking population the diet is the main source of cadmium exposure, often accounting for more than 90% of the cadmium intake (WHO, 1992; UNEP, 2006). The main food items that contribute to the cadmium exposure are cereals and vegetables (Olsson *et al.*, 2002; UNEP, 2006). The amount of cadmium in different agricultural crops depends on the rate of uptake from soil, which is influenced by the forms of the element, the physical-chemical properties of the soil, and the plant species (EFSA, 2009). Other food items that are known to contain high cadmium concentrations are shellfish and offal (e.g. kidney and liver) (UNEP, 2006; EFSA, 2009). In the general non-smoking population, less than 10% of the total cadmium exposure originates from ambient air (Vahter *et al.*, 1991) and drinking water (Olsson *et al.*, 2002). In smokers, the cigarette smoke is the main source of cadmium exposure and causes a significant increase in the blood cadmium concentration, although there are large variations depending on the type of tobacco (Järup *et al.*, 1998).

1.4 ADVERSE HEALTH EFFECTS

Cadmium is not essential for any biological function in humans. The first description of adverse health effects by cadmium in humans was published in the 1930's and referred to lung damage in cadmium exposed workers (see e.g. Nordberg, 2004). In the 1940's, several adverse health effects on bone, lung and kidney were reported in cadmium exposed workers (see e.g. Friberg *et al.*, 1985). Since then, numerous studies have

shown adverse health effects also in the general non-occupationally exposed population (Järup *et al.*, 1998; EFSA, 2009). In addition, there is increasing evidence that women may be at increased risk (Nishijo *et al.*, 2004; Vahter *et al.*, 2007).

1.4.1 Kidney

The critical organ for chronic cadmium exposure is considered to be the kidney and renal dysfunction the critical effect (WHO, 1992; EFSA, 2009). Cadmium accumulates in the renal cortex and induces tubular damage (Barbier *et al.*, 2005), which affects the capacity to reabsorb substances from the primary urine. Following chronic cadmium exposure a large proportion of cadmium in plasma is bound to metallothionein (MT). Since the MT-cadmium complex has a small molecular size it is efficiently filtered through the glomerulus and reabsorbed by the tubular cells. In the tubular cells, the MT-cadmium complex is eventually degraded by lysosomes and cadmium is released into the cytoplasm where it may be re-bound to MT produced by the tubular cells or bound to functional groups in the cell. The first early sign of cadmium-induced tubular damage is proteinuria, which is characterized by increased urinary excretion of low-molecular weight proteins and tubular enzymes. There are several sensitive indicators of cadmium-induced tubular proteinuria, such as β2-microglubulin, retinol binding protein (RBP), protein HC (α2-microglobulin), and the enzyme N-acetyl-β-glucosaminidase (NAG) (Järup *et al.*, 1998). If the cadmium exposure continues, the tubular damage will progress and glomerular dysfunction may also emerge, with a decreased glomerular filtration rate (GFR). This has been demonstrated in heavily exposed subjects (Roels *et al.*, 1989; Kido *et al.*, 1990; Järup *et al.*, 1995), but recently also in women with a chronic low-level environmental exposure (Åkesson *et al.*, 2005). GFR can be measured via clearance of different substances from serum, such as creatinine or cystatin C (Dharnidharka *et al.*, 2002). There is increasing evidence that individuals with diabetes may be more susceptible to cadmium-induced kidney damage (Buchet *et al.*, 1990; Åkesson *et al.*, 2005).

1.4.2 Bone

The toxic effects of cadmium on bone became evident after World War II at the outbreak of Itai-Itai disease (Japanese for Ouch-ouch disease) that occurred in certain areas of the Toyama Prefecture in Japan. Most of the Itai-Itai patients were women over 40 years of age, who had lived in the area for more than 30 years. They were affected by severe renal damage, osteomalacia, and osteoporosis, and suffered from severe pain. The source of cadmium exposure was rice, which had been irrigated with cadmium-

contaminated water from an upstream zinc mine. After further research the Japanese Ministry of Health and Welfare in 1968 concluded that "Itai-Itai disease is caused by chronic cadmium poisoning, on condition of existence of such inducing factors as pregnancy, lactation, imbalance in internal secretion, aging, and deficiency of calcium" (Kjellström *et al.*, 1986). Recent studies indicate that cadmium may be a risk factor for osteoporosis also at much lower cadmium exposure (Staessen *et al.*, 1999; Alfvén *et al.*, 2000; Honda *et al.*, 2003b; Alfvén *et al.*, 2004; Åkesson *et al.*, 2006; Gallagher *et al.*, 2008; Schutte *et al.*, 2008).

The mechanisms behind cadmium-induced bone effects are not yet fully understood. Two main hypotheses have been proposed: (*i*) an indirect effect via kidney damage (Ando *et al.*, 1978; Brzoska and Moniuszko-Jakoniuk, 2005a), and (*ii*) a direct effect on bone possibly through activation of osteoclasts, which has been indicated in a number of experimental studies (Bhattacharyya *et al.*, 1988a; Bhattacharyya *et al.*, 1988b; Wilson and Bhattacharyya, 1997). Recently, several epidemiological studies have provided evidence for a direct effect on bone also in humans (Åkesson *et al.*, 2006; Schutte *et al.*, 2008; Engström *et al.*, 2009).

1.4.3 Cancer

In 1993, the International Agency for Research on Cancer (IARC) has classified cadmium and cadmium compounds as carcinogenic to humans (group 1). The classification was based on epidemiological studies showing an association with lung cancer, and possibly prostate cancer, and animal studies where cadmium exposure was associated with tumors in various tissues, by various routes of exposure, and in several species and strains (IARC, 1993). Since this evaluation was performed, several epidemiological studies have found an association between environmental relevant doses of cadmium and cancer. In a study in Belgium, there was an association between the increased body burden of cadmium and the development of lung cancer (Nawrot *et al.*, 2006). In addition, recent studies have shown that cadmium may exert estrogenic effects and thereby increase the risk of hormone-related cancers (Johnson *et al.*, 2003; McElroy *et al.*, 2006; Åkesson *et al.*, 2008). A population-based case-control study in USA found that women with urinary cadmium concentrations above 0.58 µg/g creatinine had twice the breast cancer risk of those with urinary cadmium concentrations below 0.26 µg/g creatinine (McElroy *et al.*, 2006). A recent Swedish study found a positive dose-response association between cadmium intake (mean 15 µg/day) and risk of endometrial cancer among postmenopausal women (Åkesson *et al.*,

2008). Besides the cancer forms described above there is evidence that cadmium may play a role in testicular cancer, kidney cancer, bladder cancer, pancreatic cancer, and gall bladder cancer (Huff *et al.*, 2007). Currently, IARC is re-evaluating all human carcinogens.

1.5 GENDER DIFFERENCES

The first evidence of gender differences in relation to adverse health effects of cadmium became evident already at the outbreak of Itai-Itai as this disease almost exclusively affected elderly, multiparous women (Kjellström *et al.*, 1986). Despite this, research on gender differences in relation to adverse health effects is limited, especially at chronic low-level exposure (Nishijo *et al.*, 2004; Vahter *et al.*, 2007). In Belgium, a prospective study showed that chronic cadmium exposure was associated with decreased bone mineral density and increased risk of fractures in postmenopausal women (Staessen *et al.*, 1999). In fact, experimental studies indicate that the effects of cadmium on calcium metabolism and its regulatory hormones are more pronounced among female than male rats (Brzoska and Moniuszko-Jakoniuk, 2005a; Brzoska and Moniuszko-Jakoniuk, 2005b). A Swedish study on middle-aged women observed a cadmium-induced decrease in glomerular filtration at much lower levels than was previously observed in male workers (Åkesson *et al.*, 2005). On the contrary, evaluations on the critical level that causes cadmium-induced tubular effects do not give any clear evidence for gender differences (Jin *et al.*, 2004b; Uno *et al.*, 2005; Kobayashi *et al.*, 2006).

As discussed above cadmium has recently been shown to mimic estrogen (Johnson *et al.*, 2003) and thereby increase the risk of hormone-related cancers in women (McElroy *et al.*, 2006; Åkesson *et al.*, 2008). On the other hand, cadmium has also been shown to exert androgen-like activities both *in vitro* and *in vivo* (Takiguchi and Yoshihara, 2006) and there is some evidence for an association with prostate cancer (van Wijngaarden *et al.*, 2008).

1.6 RISK CHARACTERIZATION

Already in 1972, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a Provisional Tolerable Weekly Intake (PTWI) for cadmium of 400-500 µg per person, which corresponds to approximately 7-8 µg/kg body weight per week and 60-70 µg/day for an individual of 60 kg (FAO/WHO, 1972). The PTWI considers the life-long intake, which should only be exceeded under short periods of time in order to

avoid adverse health effects (Herrman and Younes, 1999). In 1988, JECFA performed a new evaluation of cadmium, but the conclusion was the same, i.e. that the oral intake of cadmium should not exceed 1 µg/kg body weight per day. Thus, the PTWI was kept at 7 µg/kg body weight (FAO/WHO, 1988). It has thereafter been re-evaluated several times, but was still maintained at the meeting in 2005 (FAO/WHO, 2005).

In 1992, WHO concluded that a urinary cadmium concentration of 10 µg/g creatinine, corresponding to 200 mg cadmium/kg kidney cortex, caused tubular proteinuria in 10% of the population (WHO, 1992). This estimate was however mainly based on cross-sectional studies performed in middle-aged male workers and the methods to detect kidney damage were relatively crude. During the past 20 years more sensitive methods have become available to detect kidney damage and large population groups exposed to low levels of cadmium have been examined. In 1998 Järup and coworkers published an extensive review on cadmium and concluded that cadmium-induced tubular damage occurred already at urinary cadmium concentrations of about 2.5 µg/g creatinine, corresponding to about 50 µg/g in the kidneys (Järup *et al.*, 1998). This is further supported by several later studies, which indicated that toxic effects on both kidneys and bone occur at similar and even lower urinary cadmium concentrations (Alfvén *et al.*, 2000; Järup *et al.*, 2000; Noonan *et al.*, 2002; Åkesson *et al.*, 2005; Åkesson *et al.*, 2006).

Based on recent data presented above on adverse health effects at much lower exposure levels the PTWI has been under extensive debate. First in March 2009, the European Food Safety Authority (EFSA) established a Tolerable Weekly Intake (TWI) for cadmium of 2.5 µg/kg body weight (EFSA, 2009). The TWI is close to the mean exposure for adults in Europe, and specific subgroups such as vegetarians, children, smokers and individuals resident in contaminated areas may even exceed the TWI with about 2-fold (EFSA, 2009). Therefore, the exposure at the population level should be reduced.

1.7 TOXICOKINETICS

1.7.1 Absorption from the diet

In humans, the gastrointestinal absorption of cadmium is generally low; usually less than 5% of the ingested cadmium is transported to blood (Morgan and Sherlock, 1984; WHO, 1992). However, a number of different factors may alter the absorption, such as the type of diet, the nutritional status of the individual, as well as age.

1.7.1.1 Nutritional status

Increased intestinal absorption of cadmium at low iron stores has been documented in both experimental and epidemiological studies (Flanagan *et al.*, 1978; Berglund *et al.*, 1994; Åkesson *et al.*, 2002; Olsson *et al.*, 2002; Satarug *et al.*, 2004). Experimental studies have demonstrated that the increased uptake of cadmium is mediated via the main iron transporters, the apical divalent metal transporter 1 (DMT1) and the basolateral ferroportin 1 (FPN1) (Tallkvist *et al.*, 2001; Park *et al.*, 2002; Ryu *et al.*, 2004; Kim *et al.*, 2007), which are up-regulated at low iron stores (Zoller *et al.*, 2001). Several experimental studies have also shown that low dietary intake of zinc and calcium is associated with elevated cadmium accumulation in various organs and it was proposed that cadmium may be taken up by zinc and calcium transporters (Waalkes, 1986; Min *et al.*, 2008b; Reeves and Chaney, 2008). In a recent study conducted on mice with a non-functional DMT1, the absorption of cadmium was still similar to that in wild type mice, which clearly indicates that DMT1 is not the only transporter involved in the intestinal cadmium absorption *in vivo* (Suzuki *et al.*, 2008). In fact, another recent study on mice suggested that the intestinal cadmium uptake is mediated via several different pathways that maintain the homeostasis of essential micronutrients (Min *et al.*, 2008a). However, the relevance for humans is not yet known.

1.7.1.2 Gender differences

In general, women have higher cadmium concentrations in blood, urine and kidneys compared to men (Järup *et al.*, 1998; Baecklund *et al.*, 1999; Vahter *et al.*, 2002). The main reason for the higher cadmium body burden is probably related to the increased intestinal absorption of cadmium at low iron stores (Tallkvist *et al.*, 2001; Ryu *et al.*, 2004; Kim *et al.*, 2007), which has been shown in different groups of pregnant and non-pregnant women (Berglund *et al.*, 1994; Åkesson *et al.*, 2002; Olsson *et al.*, 2002; Satarug *et al.*, 2004). Furthermore, there seems to be genetic factors involved in the toxicokinetics of cadmium. Twin studies have shown a significantly higher genetic influence on blood cadmium concentrations in women (65%) compared to men (13%) (Björkman *et al.*, 2000). Possibly, this genetic difference is at least partly related to the similar blood losses that homozygous female twins have during menstruations and thereby similar intestinal absorption of iron and cadmium compared to heterozygous twins. The importance of increased cadmium absorption at low iron stores in women is further supported by the fact that the difference in blood cadmium concentrations between men and women becomes less evident or even non-existing after menopause (Baecklund *et al.*, 1999).

1.7.1.3 Type of diet

Rice is known to easily take up cadmium from soil (Tsukahara *et al.*, 2003; Simmons *et al.*, 2005), making it an important source of cadmium exposure. In many parts of the world rice is an important staple food, which is eaten two to three times per day (Sudo *et al.*, 2004; Williams *et al.*, 2005; Rahman *et al.*, 2009). Polished rice is known to contain very low concentrations of iron and zinc (Gregorio et al. 2000; Graham et al. 2007) and, therefore, anemia and low zinc status are common in rice-eating populations (Ross et al. 2002). This may, in turn, increase the absorption of cadmium from rice.

Chronic cadmium exposure via polluted rice has been associated with adverse health effects in both China and Japan (Jin *et al.*, 2004a; Nogawa *et al.*, 2004), and in Japan even increased mortality (Nogawa *et al.*, 2004). On the other hand, diets that are rich in shellfish may contain high concentrations of cadmium, but are not as clearly associated with cadmium-induced adverse health effects as the rice-based diet (McKenzie-Parnell *et al.*, 1988). Vahter and coworkers compared the bioavailability of cadmium from a mixed diet (low in shellfish) to that from a shellfish diet (once a week or more) and found no significant difference in the cadmium concentrations in urine and blood, even though the shellfish diet contained twice as much cadmium as the mixed diet (Vahter *et al.*, 1996). Part of this could be explained by the much higher iron stores in the women in the shellfish group compared to the women in the mixed diet group. These results indicate that there are certain differences in the bioavailability of dietary cadmium in relation to the type of diet some of which may be related to nutritional status.

1.7.1.4 Increased absorption in infants

Young children have a higher intestinal absorption of cadmium compared to adults (usually less than 5%). In a study by Crews and coworkers, 12 month old infants were fed porridge from ^{106}Cd labeled wheat for breakfast during two consecutive days and feces were collected for four days following consumption of the first test meal (Crews *et al.*, 2000). The intestinal absorption of cadmium varied from 4 to 37% with a mean of 18%. In addition, the mechanisms of cadmium absorption appear to be different. In particular, the iron regulation is immature at a young age and iron supplementation of infants increased iron absorption independent of iron status (Domellöf *et al.*, 2001). This is further supported by a study in suckling piglets, where the uptake of cadmium was not higher in those who were iron-deficient and the expression and localization of DMT1 and FPN1 was not affected by iron status (Öhrvik *et al.*, 2007).

1.7.2 Distribution and excretion

Following chronic cadmium exposure there is a slow release of cadmium-metallothionein (MT) complexes from the intestine and the liver to blood (Nordberg *et al.*, 1986; Muller *et al.*, 1986). Metallothionein is a low-molecular weight cysteine-rich protein, which binds cadmium as well as essential metals like zinc and copper (Klaassen *et al.*, 1999). In addition, cadmium can bind to other sulfhydryl-rich low-molecular weight peptides or amino acids (Zalups and Ahmad, 2003). The cadmium-MT complex in plasma is, due to its low molecular weight, efficiently filtered through the glomerulus and then reabsorbed by the tubular cells (Nordberg *et al.*, 1986; Järup *et al.*, 1998). In adults from industrialized countries, cadmium has been detected in almost all tissues, but the highest concentrations are found in the liver and kidneys (Sumino *et al.*, 1975; Chung *et al.*, 1986). At birth the cadmium concentration in the kidney is close to zero (Lutz *et al.*, 1996) and then it increases with age to peak around 50 to 60 years of age, after which it start to decline (Elinder *et al.*, 1976; Chung *et al.*, 1986). Cadmium is excreted very slowly from the human body and the half-time in the kidney is 10-30 years (Järup *et al.*, 1998).

1.8 FEMALE REPRODUCTION AND EARLY LIFE EXPOSURE

Recently, cadmium in environmental relevant doses was shown to affect the female reproduction system by inducing several estrogenic responses in ovariectomized animals, such as increased uterine weight, growth and further development of the mammary glands, and induction of hormone-regulated genes both in the uterine and mammary gland tissues (Johnson *et al.*, 2003). In humans, the estrogenic effects of cadmium have been related to increased risk of hormone-related cancers in women, as described in more detail above.

Cadmium in known to accumulate in the human placenta and only small amounts pass over to the fetus (Korpela *et al.*, 1986; Osman *et al.*, 2000). In smokers, cadmium in placenta was negatively associated with the levels of progesterone (Piasek *et al.*, 2001) and *in vitro* experiments have indicated a dose-dependent decrease in human chorionic gonadotropin following cadmium exposure (Wier *et al.*, 1990; McAleer and Tuan, 2001). This may result in negative pregnancy outcomes. In animal studies, *in utero* exposure to a low dose of cadmium was found to mimic estrogen and induce earlier onset of puberty and changes in the mammary gland of female offspring (Johnson *et al.*, 2003). In humans, a negative association has been found between umbilical cord blood cadmium concentrations and birth weight (Galicia-Garcia *et al.*, 1997; Salpietro

et al., 2002). Also, maternal cadmium exposure was associated with decreased birth length (Zhang *et al.*, 2004b) as well as preterm delivery (Nishijo *et al.*, 2002).

In general, the transfer of cadmium to breast milk is low (Hallen *et al.*, 1995). Still, experimental studies have shown that cadmium exposure via milk may cause neurotoxicity in the offspring (Andersson *et al.*, 1997; Petersson Grawé *et al.*, 2004).

In conclusion, the data presented above indicate that pregnant women and their offspring are sensitive groups for cadmium exposure and more studies should focus on the cadmium exposure and associated toxicity in these populations.

2 AIMS OF THE THESIS

The overall aim of the present thesis was to investigate the interactions between cadmium and essential micronutrients in pregnant and lactating women.
The more specific aims were to elucidate:

- ✓ The cadmium exposure in women in a rural area of Bangladesh where rice is the main staple food and malnutrition is prevalent.

- ✓ The influence of nutritional factors on the uptake and accumulation of cadmium.

- ✓ The influence of pregnancy on the uptake and accumulation of cadmium.

- ✓ The exposure to cadmium *in utero* and via breast milk.

3 MATERIALS AND METHODS

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

3.1 STUDY AREA AND POPULATION

3.1.1 Matlab, Bangladesh

The study area was Matlab, which is a rural area located about 53 km southeast of Dhaka, the capital city of Bangladesh. Since 1966, the International Centre of Diarrhoeal Disease Research, Bangladesh (ICDDR,B) has been running a large-scale Health and Demographic Surveillance System (HDSS) in this area that covers a population of about 220 000 individuals. The HDSS is updated continuously with information collected by community health research workers who visit every household in the area on a monthly basis and records all vital events (e.g. marriages, births, deaths, in- and out-migration). In one part of the HDSS area (about 110 000 inhabitants), ICDDR,B provides health services to the inhabitants via a central hospital and four connected sub-centers. The studies in the present thesis (**Paper I-IV**) have been performed in the ICCRD,B service area, which covers four blocks in Matlab called A, B, C and D.

3.1.2 Participants

The studies in the present thesis were nested into a population-based, randomized food and micronutrient supplementation trial called Maternal and Infant Nutrition Interventions of Matlab (MINIMat). All women who were resident in the ICDDR,B service area and who became pregnant from November 2001 to October 2003 were invited to participate in this trial. The MINIMat trial included a 2x3 factorial design; two food supplementation groups that were subdivided into three different micronutrient supplementation groups, resulting in six groups. The women were allocated either to promotion of early start of the food supplementation, immediately after the pregnancy was identified, or to start at the time of their own choice (usual start). The micronutrient supplementations, to which all women were randomly assigned, started in gestational week 14 and continued daily until three months post partum. The three different types of micronutrient supplementations contained either (*i*) 30 mg iron and 400 µg folic acid, or (*ii*) 60 mg iron and 400 µg folic acid (usual care), or (*iii*) 30 mg iron and 400 µg folic acid as well as the Recommended Dietary

Allowance (RDA) of 13 additional vitamins and minerals (UNICEF/WHO/UNU, 1999).

For the purpose of the studies conducted in **Paper I-III**, 1000 women were randomly selected from the 2119 women who were enrolled from January to December 2002. In total, 752 of these 1000 randomly selected women were followed up to six months post partum. The participants in **Paper IV** consisted of women that had been recruited to MINIMat Phase II. These women had participated in the original MINIMat trial, but were identified as pregnant once again at the monthly routine HDSS visit between March 2004 and May 2007 and were thereby invited to participate in MINIMat Phase II (subsequent pregnancy). In total, 692 women were enrolled in MINIMat Phase II.

3.2 SAMPLING AND DATA COLLECTION

The present studies have included several different biological samples collected in different gestational weeks, at delivery, and during lactation. A summary of the different study designs and sample collections is presented in **Table 1**. The maternal cadmium exposure was assessed both via measurement of cadmium in urine, which reflects the body burden, and in blood (erythrocyte fraction), reflecting the current exposure. The cadmium concentration in the erythrocyte fraction is equivalent to whole blood as an exposure marker, as cadmium in blood is present almost entirely in the

Table 1. Summary of the different study designs and sample collections.

Paper	Samples	Time	Analyses (number of samples, n)
I	Urine	GW8	Cd (890)
	Plasma	GW14	p-Ft (737), p-Zn (741)
II	Erythrocytes	GW14	Cd, Mg, Ca, Mn, Cu, Zn, Se (408)
		6 mo pp	Cd (281)
	Plasma	GW14	p-Ft, p-Zn (404)
		6 mo pp	p-Ft, p-Zn (209)
III	Urine	GW8	Cd (121)
	Erythrocytes	6 mo pp	Cd, Mg, Ca, Mn, Cu, Zn, Se (123)
	Plasma	GW14	p-Ft, p-Zn (114)
		6 mo pp	p-Ft, p-Zn (77)
	Breast milk	2 mo pp	Cd, Mg, Ca, Mn, Fe, Cu, Zn, Se (123)
IV	Urine	GW8	Cd (41)
	Placenta	Delivery	Cd, Mg, Ca, Mn, Cu, Zn, Se (44)
	Cord erythrocytes	Delivery	Cd, Mg, Ca, Mn, Fe, Cu, Zn, Se (41)

GW, gestational week; mo pp, months post partum

erythrocytes (Järup *et al.*, 1998). Besides cadmium, several nutritional markers were measured in maternal plasma (ferritin (p-Ft) and zinc (p-Zn)) and in erythrocytes (magnesium, calcium, manganese, copper, zinc and selenium). The cadmium exposure and interactions with essential micronutrients during early life was assessed via measurements in placental tissue, corresponding umbilical cord erythrocytes, and breast milk.

Spot urine samples were collected in the women's homes immediately after pregnancy was identified via a urine test, usually around gestational week eight. Blood samples were collected at the health clinics in gestational week 14, as well as six months post partum. The placentas and umbilical cord blood samples were collected immediately after delivery from women giving birth at the central hospital or any of the four connected sub-centers. Breast milk was collected two months post partum as spot samples, expressed manually. After collection, all the samples were stored in -70ºC (urine and plasma) or -20ºC (maternal and umbilical cord erythrocytes, placentas, and breast milk) freezers at the Matlab hospital awaiting further transport and analyses.

The personal characteristics were obtained via different questionnaires in the MINIMat trail. The women's smoking habits were recorded after delivery. The question was if they had smoked bidi and/or cigarettes, and if so how many times per day, during their last pregnancy. Socio-economic status was calculated using a wealth index, which was created based on information regarding household assets such as land, construction material of the walls of the house, ownership of household utensils (e.g. radio, television, electric fan, table and chairs), number of clothes (e.g. sarees and shalwar-kameez), and number of shoes and sandals (Gwatkin *et al.*, 2000). In the statistical analyses, socio-economic status was included as a continuous variable, while in the presentation of the women's personal characteristics it was divided into quintiles (one representing those with the lowest and five those with the highest socio-economic status). The women's height and weight were collected and used to calculate the body mass index (BMI; kg/m^2) in early pregnancy (usually around gestational week eight).

3.3 ICPMS ANALYSES

The sample preparations and analyses of cadmium and other elements were performed at the Institute of Environmental Medicine at the Karolinska Institutet in Stockholm, Sweden.

3.3.1 Sample preparation

The urine samples were brought to room temperature, vigorously shaken and prepared for element analysis via simple dilution with 1% nitric acid. The other samples (erythrocytes, placenta and breast milk) were digested before element analyses using a Milestone ultraCLAVE II microwave digestion system (EMLS, Leutkirch, Germany). Placentas were thawed and both the decidua basalis and the chorionic plate were removed, leaving only the trophoblastic tissue. Three sub-samples of placental tissue were cut out, starting from the peri-insertional region, close to the umbilical cord and continuing outwards. Thereafter, each sub-sample was freeze-dried (EF4 Modulyo Freeze Dryer, Edwards High Vacuum, Sussex, United Kingdom). Approximately 0.5 g sample was used for the digestion. Erythrocytes and breast milk were mixed with 2 mL of nitric acid (65% suprapur, Merck, Darmstadt, Germany) and 3 mL of deionized water, while the dried placental tissue was mixed with 5 mL nitric acid only. The autoclave was loaded with $40*10^6$ Pa nitrogen gas and thereafter heated to 250ºC for 30 minutes. The digested, essentially carbon-free solutions were then transferred to acid-washed polyethylene tubes and diluted with deionized water to a nitric acid concentration of 20%. In each run, we also included appropriate reference materials and three blanks.

3.3.2 Analysis of cadmium in urine

Urinary cadmium was measured using inductively coupled plasma mass spectrometry (ICPMS; Agilent 7500ce, Agilent Technologies, Waldbronn, Germany) with a collision/reaction cell system, autosampler (Cetac ASX-510), integrated sample introduction system (ISIS), and a MicroMist nebulizer in quartz (**Paper I** and **III**). In order to minimize possible interferences, the ICPMS was operated in helium-mode and four cadmium isotopes (*m/z* 111, 112, 113 and 114) with appropriate interference corrections were monitored. There was a very good agreement between isotope 111 and an average of the four isotopes (**Figure 1**) and therefore, the urinary cadmium concentrations presented are based on an average of the four isotopes. In **Paper IV**, the same ICPMS method was used for measuring urinary cadmium, but with minor modifications. The ICPMS was no longer equipped with ISIS, and the nebulizer had been changed to an Agilent Micro Flow nebulizer. In addition, only isotope 111 was monitored in helium-mode without any additional mathematical interference corrections.

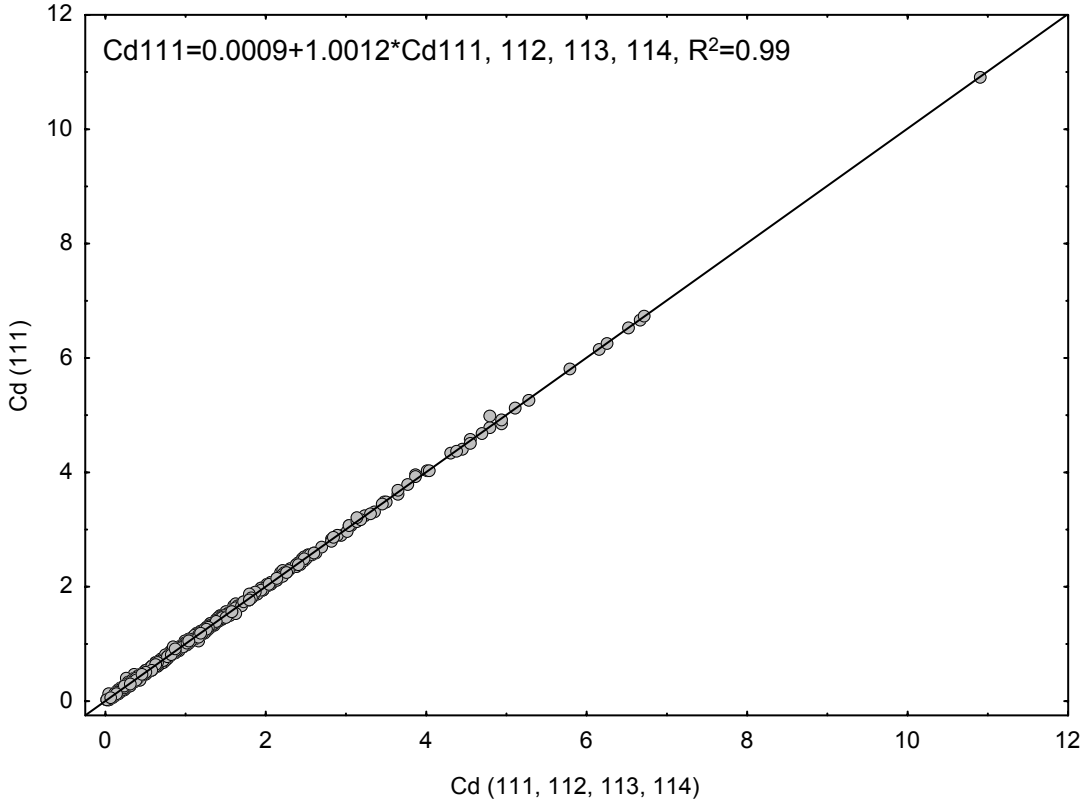

Figure 1. Comparison between cadmium 111 and a mean of the four cadmium isotopes (111, 112, 113, 114).

3.3.3 Urinary adjustments

Timed collection, such as 24 hour collection, is the optimal way to measure excretion of different biomarkers in urine, but because of the difficulties involved in this type of collection, especially in large cohort studies (Johansson *et al.*, 1999), we have relied on concentrations in spot urine samples. Spot urine samples vary considerably in dilution, caused by variation in fluid intake, physical activity, temperature etc, which needs to be compensated for in order to avoid potential bias. A common way of compensating for this variation in dilution is to adjust for the urinary creatinine concentration, since the rate of creatinine excretion is fairly constant in human urine over time. However, creatinine is formed by creatine, which is highly associated with muscle mass, and present in meat and therefore, creatinine varies by age, body size and diet (Boeniger *et al.*, 1993). Another way of compensating for variation in dilution in spot urine samples is to adjust for specific gravity, which previously has been found to be less dependent on factors such as body size, age, and gender than urinary creatinine excretion (Nermell *et al.*, 2008). The specific gravity is measured with a refractometer as the angle of light refraction between air and urine sample, and this refraction increases with the total solute concentration in urine (George, 2001).

In the present studies (**Paper I, III,** and **IV**), which include women with a wide range of nutritional status, the variation in dilution was compensated for by adjusting to the mean specific gravity (1.012 g/mL; URICON-NE refractometer, Atago Co., Ltd, Tokyo, Japan), which previously has been found to be less dependent on nutrition than urinary creatinine excretion in the present population (Nermell *et al.*, 2008). However, to confirm those results in the present cohort, urinary creatinine (g/L) was measured in a sub-sample of 420 women, by the Jaffe method (Hare, 1950). The mean creatinine concentration was 0.57 g/L and only 5% of the women had values above 1 g/L. In the 420 studied women the mean urinary cadmium concentration of 0.76 µg/L, adjusted for the mean specific gravity of 1.012 g/mL, corresponds to 1.4 µg cadmium/g creatinine. In addition, urinary creatinine was found to be highly associated with BMI ($p<0.001$), in line with previous findings (Suwazono *et al.*, 2005; Nermell *et al.*, 2008). Thus, if the urinary creatinine concentrations are not considered the urinary cadmium concentrations may be over-estimated among malnourished women.

3.3.4 Analysis of elements in blood, placenta and breast milk

In **paper II** and **III**, digested samples of erythrocytes and breast milk were measured for various elements using inductively coupled plasma mass spectrometry (ICPMS; Agilent 7500ce, Agilent Technologies, Waldbronn, Germany). The ICPMS was equipped with a collision/reaction cell system, autosampler (Cetac ASX-510), and a quartz MicroMist nebulizer. The following elements were measured: magnesium *m/z* 25, calcium *m/z* 43, manganese *m/*z 55, iron *m/z* 56 (only in breast milk and umbilical cord erythrocytes), copper *m/*z 65, zinc *m/*z 66, selenium *m/z* 78, and cadmium *m/*z 111. All the elements were measured in helium-mode except selenium, which was measured in hydrogen-mode. In **paper IV**, the same elements were measured in digested umbilical cord erythrocytes and placental tissue. During these measurements the ICPMS was no longer equipped with ISIS, and the nebulizer had been changed to an Agilent Micro Flow nebulizer.

3.3.5 Quality control

In order to control the analytical accuracy, appropriate reference materials were included in each analysis. For cadmium, the results of the different types of reference materials included in **Paper I-IV** are presented in **Table 2**. For the quality control results of the essential micronutrients measured with ICPMS, the reader is referred to each individual paper.

In general, our obtained values showed satisfactory agreement with the reference values. The cadmium concentrations in Seronorm NO2525 (Seronorm^{TM} Trace Elements Urine, REF 201205, SERO AS, Billingstad, Norway) were somewhat lower than the recommended value, but similar results have been obtained in other studies using ICP-SFMS (Ellingsen *et al.*, 2006). In **paper III**, reference materials for whole blood were used for quality control of both erythrocytes and breast milk. The main reason for not including a separate reference material for breast milk was that we did not find a product that contained both cadmium and all the essential micronutrients that we wanted to measure. However, as all the samples were digested prior to the ICPMS analyses the possibility of matrix interferences had been reduced substantially. Reference materials for kidney and liver (CRM 185 bovine liver and CRM 186 pig kidney, Community Bureau of Reference (BCR), Brussels, Belgium) were used for quality control of placental tissue (**Paper IV**).

Table 2. Obtained values (mean±SD) of reference materials used for analytical quality control of cadmium in urine, erythrocytes, breast milk and placental tissue.

Paper	Reference materials	n	Obtained value[1]	Reference value[1]
I	Seronorm NO2524 **Urine**	47	0.12±0.02	0.11±0.1
	Seronorm NO2525 **Urine**	83	4.5±0.22	5.06±0.22
II	Seronorm MR4206 **Whole blood**	38	0.71±0.09	0.74±0.06
	Seronorm 0503109 **Whole blood**	39	6.5±0.4	6.0±0.4
	Seronorm MI0181 **Serum**	3	87±3	83±6
III	Seronorm MR4206 **Whole blood**	29	0.68±0.097	0.74±0.06
	Seronorm 0503109 **Whole blood**	28	6.5±0.38	5.1±2.3
IV	Seronorm MR4206 **Whole blood**	6	0.72±0.03	0.74±0.06
	CRM 185 **Bovine liver**	8	334±3	298±25
	CRM 186 **Pig kidney**	8	2533±84	2710±150
	Seronorm OK4636 **Urine**	6	0.30±0.01	0.31±0.05

[1]Unit µg/L

An intra-laboratory comparison for additional control of the ICPMS method was performed in **Paper I**. In this intra-laboratory comparison, a sub-set of 359 urine samples were analyzed using both Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS; Perkin Elmer Model 5000 Zeeman with HGA-500) and ICPMS. There was a good agreement between the two methods (**Figure 2**).

The limit of detection (LOD) was always calculated as three times the standard deviation (SD) of the blank values. The mean LOD for cadmium was 0.06 µg/L (range

0.02-0.1 µg/L; **Paper I-IV**). The highest LOD for cadmium (0.1 µg/L) was obtained during the analyses of digested erythrocytes, probably due to increasing background with the high nitric acid concentration of 20%. Since the transfer of cadmium to breast milk was low, 6% of the breast milk samples had cadmium concentrations below LOD (**Paper III**). Although the concentrations below LOD are measured with a larger uncertainty than those above, the exact values have been used in the calculations in an attempt to avoid distorting the true distribution. However, excluding the samples below LOD did not alter the main findings in **Paper III**.

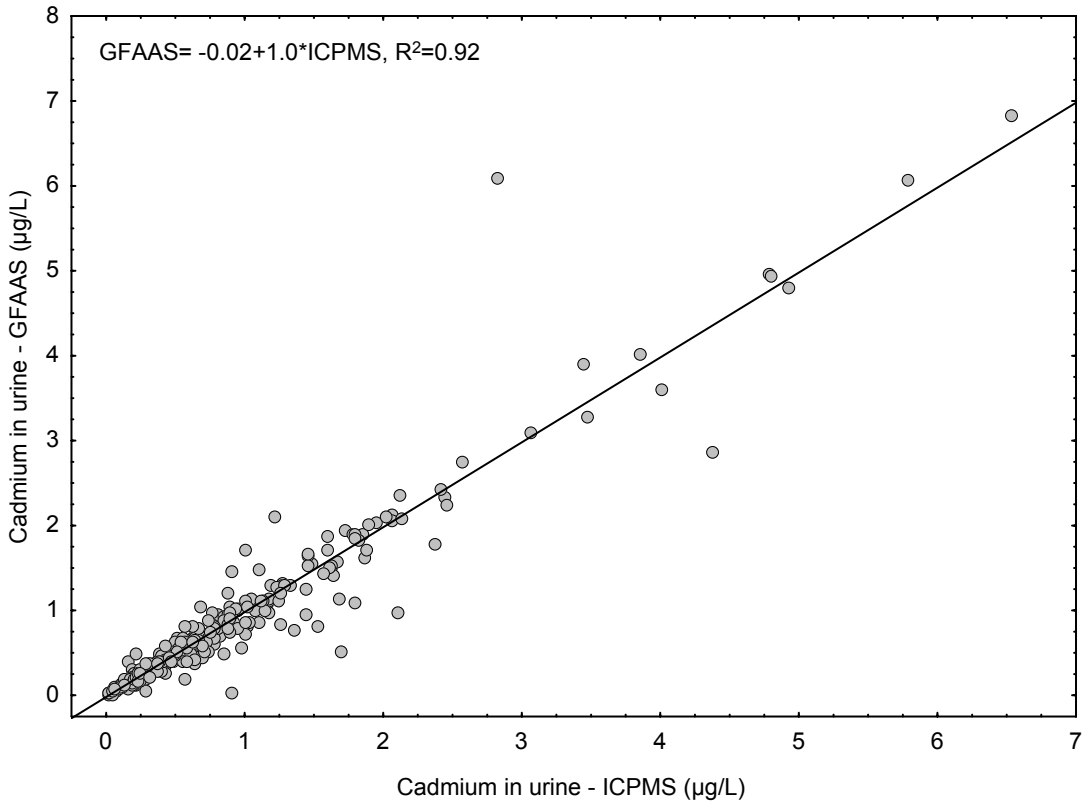

Figure 2. Intra-laboratory comparison between GFAAS and ICPMS.

3.4 NUTRITIONAL MARKERS IN PLASMA

Both ferritin and zinc in plasma were measured at the University of California, Davis, USA. Ferritin was measured using radioimmunoassay (Diagnostic Products, San Diego, CA, USA) and for quality control purposes Lyphochek® Immunoassay Plus Control (Bio-Rad Laboratories, USA) was used. Ferritin values below 20 µg/L were regarded to reflect low iron stores (Institute of Medicine, 1993), previously shown to be associated with increasing cadmium uptake and accumulation (Berglund *et al.*, 1994). Zinc was assessed by atomic absorption spectrometry (Clegg *et al.*, 1981) and quality control was maintained via a standard reference material (SRM 1577b, Bovine Liver,

National Institute of Standards and Technology, USA). The cut-off level for zinc deficiency in pregnant women, second trimester, was set to 0.50 mg/L, while at six months post partum the cut-off level for zinc deficiency was set to 0.66 mg/L in plasma (Hotz *et al.*, 2003).

3.5 WESTERN BLOT

The MT protein expression in trophoblastic placenta tissue was quantified with Western blot at the Swedish University of Agricultural Sciences, Uppsala, Sweden. Approximately 150 mg frozen trophoblastic tissue was homogenized in lysis buffer and the isolated proteins were quantified using the bicinchoninic acid protein assay (Sigma-Aldrich, Stockholm, Sweden). Aliquots of 30 µg proteins were separated on 18% Tris-HCl polyacrylamid gels under reducing conditions (Eklund *et al.*, 2004). Separated proteins were electroblotted to nitrocellulose membranes, which were blocked and then incubated with primary MT antibodies (E9, Dako; Stockholm, Sweden) followed by incubation with a secondary horse-radish-peroxidase (HRP)-conjugated antibodies (rabbit anti-mouse, P260, Dako). After each step, the blots were rinsed with PBS containing 0.5% Tween-20. HRP was detected by Enhanced Chemiluminescence Western Blotting Detection reagents (ECL Plus, Amersham Biosciences). The Chemi-Doc Gel Quantification System and Quantity-One software (BioRad) was applied to detect and quantify the intensities of the obtained bands.

3.6 ETHICAL CONSIDERATIONS

Informed consent, both oral and written was obtained from all the women about their participation in the present studies. The studies have been approved by the Research Review Committee and Ethical Review Committee at ICDDR,B, and the Regional Ethics Committee at Karolinska Institutet in Stockholm, Sweden. The results on cadmium in urine and blood have not been reported to the individual women. However, the results have been reported to ICDDR,B.

3.7 STATISTICAL ANALYSES

The statistical analyses were performed with Statistica 7.1 for Windows (StatSoft. Inc., Tulsa, OK, USA). Spearman's rank correlation test (r_s) or Kendall's Tau (r_τ) was used when testing bivariate associations between continues variables. Non-parametric tests, Mann Whitney U-Test and Kruskal Wallis test were used to test for bivariate differences between independent groups. Friedman analysis of variance (ANOVA) was used to test for differences between multiple dependent groups. In the multivariate

analyses, variables were ln (natural logarithm) transformed, to meet the requirement of equal variance and normal distribution of residuals. Tests for collinearity (tolerance and variance inflation factors) were performed and if the collinearity was unacceptable the independent variable given the highest R square of the model was chosen. Multiple linear regression analysis was used for continuous variables, while analysis of covariance (ANCOVA) was used to test for differences and possible interactions between independent groups, adjusting for covariates. Repeated ANCOVA was used to test for differences and possible interactions between dependent samples. In the multivariate analyses, only variables that were significantly associated with the dependent variable in the bivariate analyses, or changed the estimates in the model more than 10%, or had previous scientific evidence were included. The statistical significance level, including interactions, was set to $p<0.05$.

4 RESULTS AND DISCUSSION

In this section, the main results from the present thesis are summarized and discussed, and a conclusion is drawn from these results. In addition, some unpublished data is presented. For further details, the reader is referred to each individual paper (**I-IV**).

4.1 MATERNAL CADMIUM EXPOSURE

4.1.1 Biological monitoring

4.1.1.1 Cadmium in urine and blood

The long-term maternal cadmium exposure was assessed by analyzing cadmium in urine (**Paper I**), while cadmium in blood (erythrocytes fraction; **Paper II**) was used for the assessment of the current exposure. The cadmium concentration in the erythrocyte fraction is equivalent to whole blood as an exposure marker, since cadmium in blood is present almost entirely in the erythrocytes (Järup *et al.*, 1998).

In total, 890 out of 1000 randomly selected women recruited to the MINIMat trial in 2002 had provided a urine sample in early pregnancy (usually in gestational week eight; **Paper I**). In **Paper II**, the first 500 of the randomly selected women were selected for measurements of cadmium in blood (gestational week 14). In total, 408 out of the 500 selected women had provided a blood sample. A large variation was seen both in the urinary and blood cadmium concentrations (**Table 3**), and they were both positively skewed. There was a significant positive association between cadmium in urine and blood (r_s=0.50; p<0.001; n=363), indicating that the body burden influences the cadmium concentration in blood to some extent.

Table 3. The maternal cadmium concentrations in urine and blood (erythrocyte fraction).

	Urinary cadmium (µg/L)[1]	**Erythrocyte cadmium (µg/kg)**
Median	0.59	1.1
Mean	0.78	1.3
Min	0.050	0.30
10^{th} percentile	0.22	0.64
90^{th} percentile	1.5	2.3
Max	5.3	5.4
n	890	408

[1]Adjusted to the mean specific gravity of 1.012 g/mL

The median cadmium concentration in blood was 1.1 µg/kg (**Paper II**), which would correspond to approximately 0.5 µg/L in whole blood, assuming a hematocrit of 42% and that about 90 to 95% of the cadmium in whole blood is present in the erythrocyte fraction. This blood cadmium concentration is more than 2-fold higher than that found in Swedish women in early gestation (gestational week 11) (Åkesson *et al.*, 2002). The median cadmium concentration in urine was 0.59 µg/L (**Paper I**), which is about 2-fold higher than that reported for women in Sweden and USA of corresponding age (Åkesson *et al.*, 2002; McElroy *et al.*, 2007). In fact, the urinary cadmium concentrations are more similar to those among much older Swedish women, aged 53-64 years, in whom the urinary cadmium concentrations were associated with adverse health effects on both kidneys and bone (Åkesson *et al.*, 2005; Åkesson *et al.*, 2006).

4.1.1.2 Smoking habits

Smoking is an important source of cadmium exposure (WHO, 1992; Järup *et al.*, 1998). In the present studies, the women were asked after delivery if they had smoked bidi and/or cigarettes during pregnancy. All of the women had reported themselves as none-smokers in **Paper III** and **IV**. In **Paper I**, data on smoking habits were not included, but 81% of the 890 women had reported their smoking habits (none did smoke). However, women who had not responded did not have different urinary cadmium concentrations (median 0.56 µg/L; n=172) compared to those who reported that they had not smoked during pregnancy (median 0.60 µg/L; n=718; p=0.8). Similarly, in **Paper II** 9% of the women had not responded, and they did not have significantly higher blood cadmium concentrations compared to those who reported themselves as non-smokers (p=0.3). In accordance, a parallel study on arsenic exposure in both men and women reported that essentially no adult woman (aged 5th to 95th percentile; 22 to 71 years) in this rural area smoke cigarettes (Lindberg *et al.*, 2008).

4.1.1.3 Dietary intake

In the general non-smoking population, the diet is the most important source of cadmium exposure (WHO, 1992; EFSA 2009). In Bangladesh, similar to many other Asian countries, rice is the main staple food that is normally eaten several times a day (Sudo *et al.*, 2004; Williams *et al.*, 2005; Rahman *et al.*, 2009). Rice is known to easily take up cadmium from soil (Tsukahara *et al.*, 2003; Simmons *et al.*, 2005) and has been shown to be the major source of cadmium exposure in other rice-eating populations (Shimbo, 2000; Watanabe *et al.*, 2000), suggesting that it may be the primary source of cadmium also in the present study.

Data on the cadmium content in rice that is grown in Bangladesh is very limited. About 20 to 30 years ago, the total daily intake of cadmium via rice in Bangladesh was estimated to 13 µg based on measurements of cadmium in a limited number of rice samples (Masironi *et al.*, 1977; Rivai *et al.*, 1990). Since then, however, the usage of fertilizers that may be contaminated with cadmium has increased extensively in Bangladesh (UNEP, 2001), but there is little information on cadmium contamination of the fertilizers used. One report indicated concentrations of 0.5 to 3.5 mg/kg in six different fertilizers that are commonly used in Bangladesh (Rahaman *et al.*, 2007), but further analyses are needed. Thus, it is likely that the cadmium concentration in agricultural soil and thereby also the accumulation in crops have increased with the increasing use of fertilizers. This is supported by the obtained cadmium concentrations in urine (**Paper I**), as a urinary cadmium concentration of 0.59 µg/L would correspond to a daily intake of 15 to 25 µg cadmium per day (Moon *et al.*, 2003; Ikeda *et al.*, 2006).

The cadmium concentration in the tube-well water used during pregnancy was low (median 0.08 µg/L; n=265; **Paper II**), which further supports the hypothesis that food is the main source of cadmium exposure. As mentioned above, essentially no adult women in Matlab smoke cigarettes or bidi, but it is more common that they use chewing tobacco, locally called shada or zarda (Choudhury *et al.*, 2007; Lindberg *et al.*, 2008). In order to estimate the contribution from chewing tobacco, we measured cadmium in six different samples of chewing tobacco from the local markets in Matlab, and found a mean cadmium concentration of 1.3 µg/g. Therefore, chewing 1 g tobacco two to three times per day would result in ingestion of a maximum of about 4 µg cadmium, which corresponds to about 20% of that ingested with the diet.

Further studies to assess the current cadmium concentrations in different food items, especially in the many various types of rice, are needed. We are currently collecting rice samples from about 50 families in Matlab and these samples will be analyzed for both toxic and essential trace elements.

4.1.1.4 Age and socio-economic status

The cadmium concentrations in both urine (r_s=0.21; p<0.001; n=890; **Paper I**) and blood (r_s=0.19; p<0.001; n=408; **Paper II**) increased with increasing age. The cadmium concentration in urine is mainly influenced by the body burden, and it is proportional to the concentration found in the kidneys (Järup *et al.*, 1998). Thus, the

increase in urinary cadmium with age was expected and is in line with several other studies (Olsson *et al.*, 2002; Moriguchi *et al.*, 2005; McElroy *et al.*, 2007). As the women in the present studies were in the age-span of 14 to 44 years there was no tendency of a peak followed by a decline in urinary cadmium, which has been detected in women above and around 60 years of age (Sartor *et al.*, 1992; Paschal *et al.*, 2000). Blood cadmium, on the other hand, generally reflects the current exposure with a half-time of two to three months (Welinder *et al.*, 1977; Järup *et al.*, 1998).

In addition, cadmium in urine (r_s=-0.18.; p<0.001; n=890; **Paper I**) and blood (r_s=-0.13; p<0.01; n=408; **Paper II**) increased with decreasing socio-economic status, also after adjusting for age. This is probably related to food habits, such as a higher intake of rice and lower intake of food items that are low in cadmium (e.g. meats, eggs, pulses, milk, fresh vegetables and fruits) among women with low socio-economic status (Sudo *et al.*, 2004). This is supported by a study conducted in southern India, which suggested that rice was the major source of cadmium in the rural population and individuals with low socio-economic status (Srikanth *et al.*, 1995). In contrast, a study in USA found that women in the highest household income category had the highest urinary cadmium concentration (McElroy *et al.*, 2007), probably reflecting other dietary habits than those of the Bangladeshi women. In the present study, poor women were also more likely to be malnourished and thereby have a higher uptake of cadmium from food (see section 4.1.2).

4.1.1.5 Geographical variations

The cadmium exposure varied significantly between the four different administrative areas of residence in Matlab, referred to as blocks (A, B, C and D). As shown in **Figure 3** the concentrations of cadmium in urine and blood display similar differences between the different blocks.

Women in block C had significantly higher concentrations than the women in the other three blocks (A, B and D), even after adjusting for age and socio-economic status. Thus, age or socio-economic status did not explain the higher cadmium exposure in Block C. Instead, the increased cadmium exposure in Block C, located furthest up north, may be related to natural variations in soil cadmium concentrations, or to more extensive usage of cadmium-contaminated fertilizers, leading to increased cadmium concentrations in locally grown rice and vegetables.

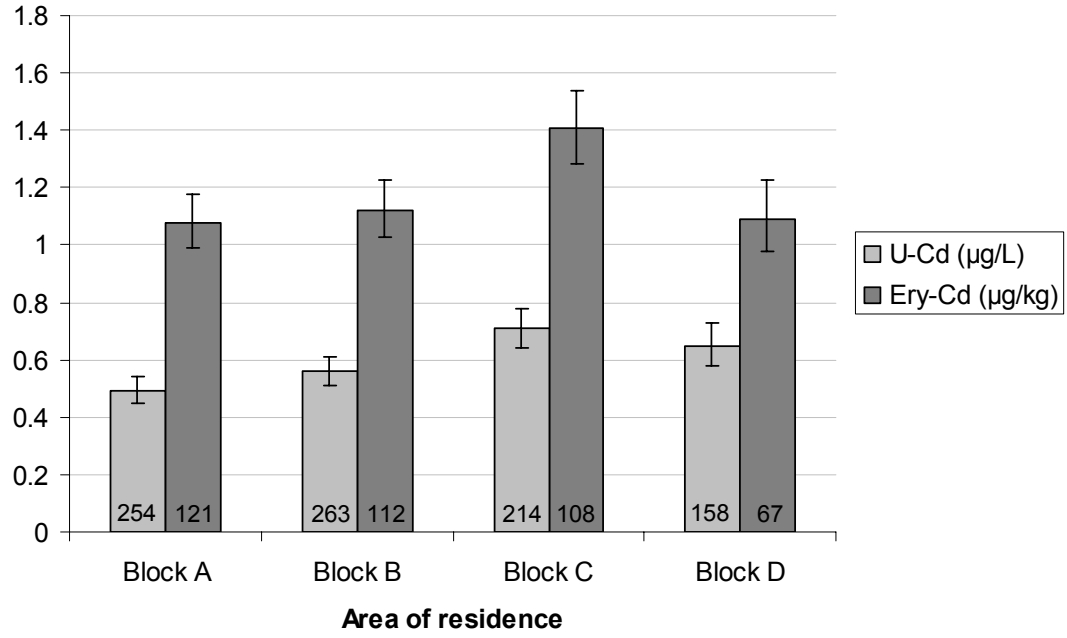

Figure 3. Geometric mean and corresponding 95% confidence intervals of cadmium in urine (U-Cd) and blood (erythrocyte fraction; Ery-Cd) by different geographical areas in Matlab, adjusted for age and socio-economic status.

4.1.1.6 Conclusion

The level of cadmium exposure among the studied pregnant Bangladeshi women was in the same range as that associated with adverse health effects on both kidneys and bone in other countries. Further assessment of the cadmium exposure, especially among malnourished women in childbearing age and potentially related adverse health effects is needed. The exposure source appears to be food, as none of the women were smokers. Therefore, it is essential to measure cadmium in different foods, in particularly various types of rice and vegetables, as well as different fertilizers.

4.1.2 Influence of nutritional factors

The gastrointestinal absorption of cadmium in adults is generally low, usually less than 5% (Morgan and Sherlock, 1984; WHO, 1992). However, the absorption has been shown to vary considerably between individuals, which can partly be explained by differences in nutritional status (Flanagan *et al.*, 1978). In the present thesis, the interactions between cadmium and several different nutritional markers (e.g. BMI, p-Ft, p-Zn, and magnesium, calcium, manganese, copper, zinc and selenium in erythrocytes) were investigated in two cross-sectional studies (**Paper I** and **II**). The main findings are presented and further discussed below.

Women with low iron stores (low p-Ft) had significantly higher cadmium concentrations in blood (**Paper II**), indicating increased intestinal uptake of cadmium via the main iron transporters, DMT1 and FPN1 (Tallkvist *et al.*, 2001; Park *et al.*, 2002; Ryu *et al.*, 2004), which are up-regulated at low iron stores (Zoller *et al.*, 2001). These findings are in accordance with previous studies conducted on women in childbearing age, as well as pregnant women (Berglund *et al.*, 1994; Åkesson *et al.*, 2002; Olsson *et al.*, 2002). Also, the urinary cadmium concentrations, reflecting the body burden of cadmium, were elevated in the women with low iron stores (**Paper I**), which probably reflect that the iron deficiency often is chronic in poor Bangladeshi women. The association between blood cadmium and p-Ft was stronger than that with urinary cadmium, as blood cadmium and p-Ft are current biomarkers and thereby more closely linked to the intestinal uptake.

A new finding in the present study is the strong positive association between cadmium and manganese in blood (r_s=0.25; p<0.001; n=408; **Paper II**). Like cadmium in blood, manganese in blood was higher in women with low iron stores (**Figure 4**). Because manganese, like iron and cadmium, is transported via DMT1 (Gunshin *et al.*, 1997), there seems to be a parallel intestinal uptake of these three metals via DMT1. The competition between these metals is probably dependent on their relative concentrations in ingested food and water (e.g. iron 10-20 mg, manganese 2.5-5.0 mg, and cadmium 0.010-0.030 mg), their kinetics and the affinity to DMT1 binding sites in the intestinal mucosa. Also, DMT1 is strictly regulated by the body's iron stores (Zoller *et al.*, 2001), leading to further up-regulation if the requirements are not met.

However, DMT1 is not the only candidate for transport of cadmium and manganese, which was clearly indicated in the multivariate analysis where manganese in blood (Beta=0.18; p<0.05) was a stronger predictor of cadmium in blood than p-Ft (Beta=-0.12; p<0.05**; Paper II**). In fact, women with high manganese concentrations in blood (cut-off at median value of 22 µg/kg) had significantly higher cadmium concentrations in blood, independent of their iron stores (defined as low <20 µg/L or adequate ≥20 µg/L). In accordance, experimental studies have identified a transport system for manganese and cadmium that appears distinct from DMT1 (Himeno *et al.*, 2002). Recently, *in vitro* studies identified two transporters, ZIP8 and ZIP14, which have high affinity for both cadmium and manganese (He *et al.*, 2006; Girijashanker *et al.*, 2008), but if they mediate intestinal transport of these metals *in vivo* remains to be elucidated.

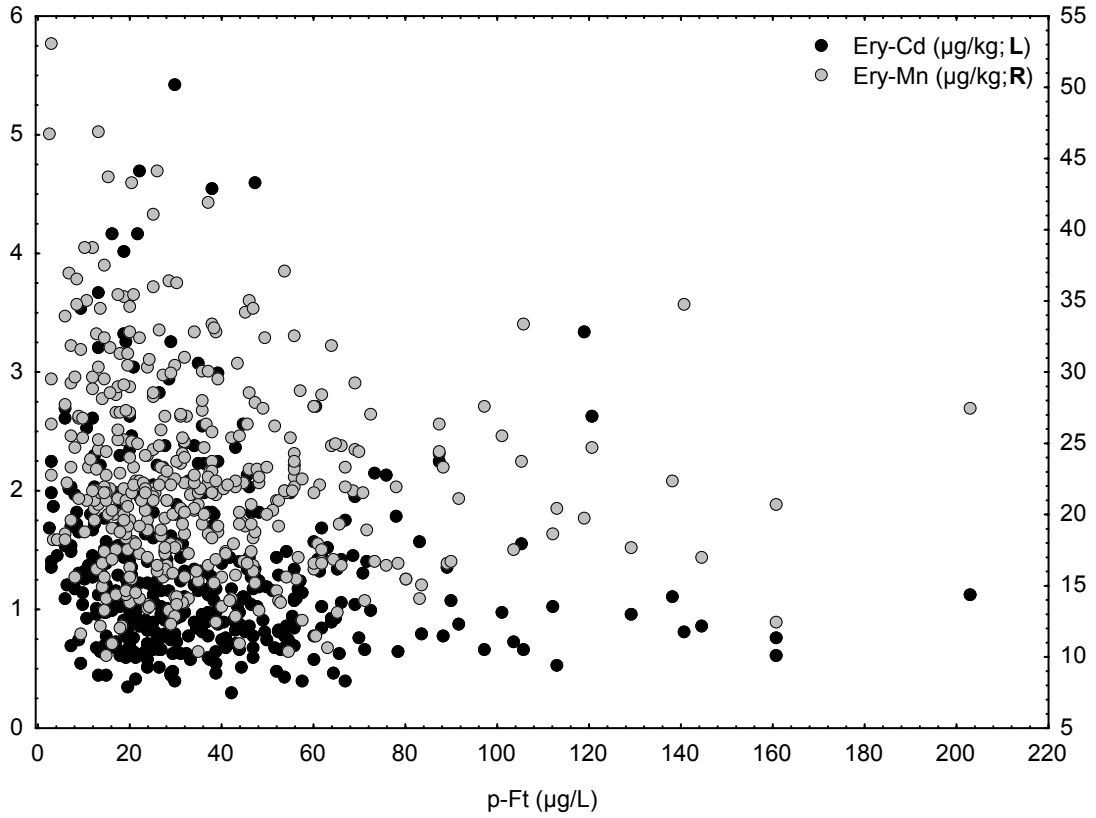

Figure 4. Association between cadmium in blood (erythrocyte fraction; Ery-Cd) and p-Ft (**Paper II**; L=Left), and between manganese in blood (Ery-Mn) and p-Ft (R=Right).

At the initiation of the present studies one of the main hypothesis was that cadmium would also be taken up by zinc transporters in the intestine and that the uptake and accumulation of cadmium would increase at low zinc status, which has previously been suggested in experimental animals with a low dietary intake of zinc (Fox *et al.*, 1984; Waalkes, 1986; Reeves and Chaney, 2002). In spite of the high prevalence of zinc deficiency in the studied women (30 to 35%; gestational week 14), we did not find any direct association between p-Zn and the cadmium concentrations in urine (**Paper I**) or blood (**Paper II**). Zinc was also measured in erythrocytes (**Paper II**), which have recently been suggested to reflect more long-term zinc deficiency (Marques *et al.*, 2007), but it was not associated with blood cadmium. Thus, the present studies did not provide any evidence for interactions at the intestinal transporter level in humans. The discrepancy towards the above mentioned experimental animal studies may be that the latter employed diets with extremely low zinc content. Several zinc transporters have been identified in the human intestine, such as the apical zinc importers ZnT5 and ZIP4 (Wang *et al.*, 2002; Dufner-Beattie *et al.*, 2003; Cragg *et al.*, 2005) and the basolateral exporter ZnT1 (McMahon and Cousins, 1998), but no studies have shown that these

transporters actually can mediate the uptake of cadmium. An *in vitro* study has shown that cadmium can induce ZnT1 mRNA levels (Langmade *et al.*, 2000).

Although no direct association was found with zinc there was a significant interaction between p-Zn and p-Ft in relation to the uptake and accumulation of cadmium. Women with low p-Ft (<20 µg/L) had significantly higher cadmium concentrations in urine (**Paper I**) and blood (**Paper II**) than women with adequate p-Ft (≥20 µg/L), but only in women with adequate p-Zn (≥0.5 mg/L), not in women with low p-Zn (<0.5 mg/L; **Figure 5**). The analyses were adjusted for age and socio-economic status. The observed interaction may be related to the fact that zinc plays an important role in the regulation of DMT1 and FPN1 in the intestine. The 5´-promoter region of DMT1 contains several metal responsive elements (MREs) (Lee *et al.*, 1998), and the metal-responsive transcription factor-1 (MTF-1), which binds to these MREs is highly regulated by zinc (Andrews, 2001). Actually, zinc has been found to increase the mRNA and the protein levels of DMT1, as well as the mRNA levels of FPN1 *in vitro* (Yamaji *et al.*, 2001). Also, in a recent *in vitro* study on intestinal crypt cells zinc treatment was shown to favor cadmium uptake (Bergeron and Jumarie, 2006).

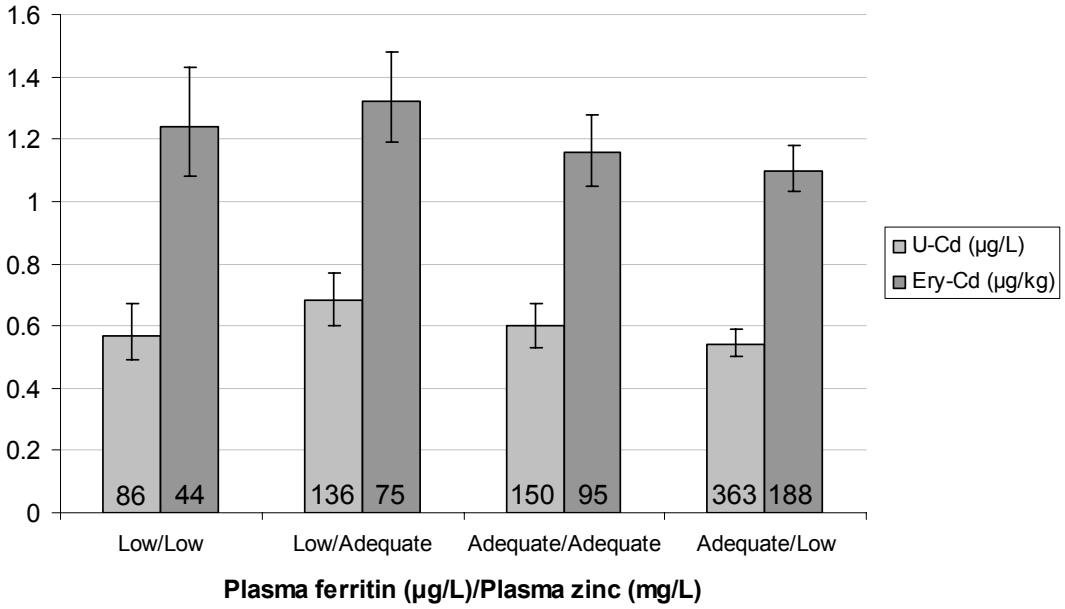

Figure 5 Geometric mean and corresponding 95% confidence intervals of cadmium in urine (U-Cd) and blood (erythrocyte fraction; Ery-Cd) by different combined levels of p-Ft (cut-off 20 µg/L) and p-Zn (cut-off 0.5 mg/L), adjusted for age and socio-economic status.

Unexpectedly, we found a strong negative association between cadmium and calcium in blood (r_s=-0.22; p<0.001; n=408; **Paper II**). In the multivariate analysis, the calcium concentration in blood (Beta=-0.11; p<0.05) explained about as much of the variation in blood cadmium as did p-Ft (Beta=-0.12; p<0.05), while manganese in blood was the strongest predictor (Beta=0.18; p<0.05). Both cadmium and calcium were measured in blood samples collected in the beginning of the second trimester (gestational week 14), which together with the first trimester is a period of increased calcium demand and thereby increased calcium absorption in the intestine (Perez *et al.*, 2008). The negative association suggests that cadmium disturbs calcium transport. Intestinal calcium uptake is mediated both via paracellular and transcellular transport. The paracellular transport occurs via diffusion through the tight junctions and is the dominant transport (>90%) at high calcium intake. The transcellular pathway across the enterocytes is mediated via different transporters and occurs predominantly (~80%) at low calcium intake (Khanal and Nemere, 2008), which is the case in Matlab, like in many other places in Bangladesh (Kimmons *et al.*, 2005; Heck *et al.*, 2007). Altogether, this suggests that the intestinal uptake of calcium in the studied women mainly occurs via the transcellular pathway. *In vitro* studies have shown that cadmium inhibits both CaT1, which transports calcium across the apical membrane of the enterocytes (Peng *et al.*, 1999), and CaATPase, which mediates the extrusion of calcium across the basolateral membrane to blood (Verbost *et al.*, 1987).

On the other hand, low dietary intake of calcium (1% of control diet) was found to increase both the expression of CaT1 and the accumulation of cadmium in the intestine, kidney and liver in mice (Min *et al.*, 2008b), suggesting that cadmium is taken up via CaT1. However, the relevance to humans is not known. Mechanistic studies to further elucidate the intestinal interactions between cadmium and calcium are highly warranted.

In conclusion, the intestinal uptake and accumulation of cadmium in early pregnancy was markedly influenced either directly or indirectly by iron, manganese and zinc. Further, cadmium seems to disturb the uptake of calcium to blood.

4.2 PREGNANCY

During pregnancy and lactation there is an increased nutritional demand for the development of the placenta and the offspring. Thus, we found it essential to assess the uptake and transport of cadmium during pregnancy, and to investigate possible

interactions with micronutrients. In the cross-sectional analyses, multiparous women had significantly higher concentrations of cadmium in urine ($p<0.001$; **Paper I**) and blood (<0.001; **Paper II**) compared to primiparous women. However, parity was also positively associated with maternal age in the present population ($p<0.001$). As age explained more of the variation in the cadmium concentrations in both urine and blood the multivariate analyses were adjusted for age and the association with parity was not explored further in the cross-sectional studies (**Paper I** and **II**).

A previous Swedish study has shown that the increase in urinary cadmium with age was more pronounced among multiparous women compared to those with one child or no children (Åkesson *et al.*, 2002), although there were only 13 women that were multiparous. We found no such trend in the Bangladeshi women (**Figure 6**).

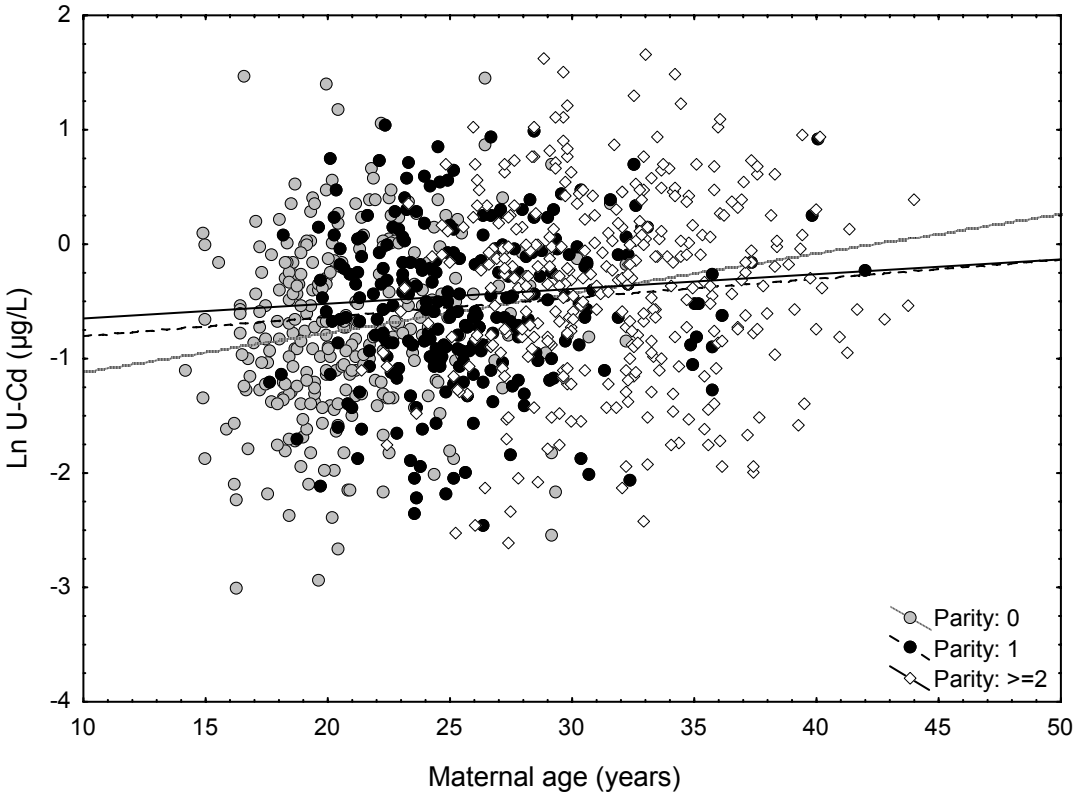

Figure 6. Urinary cadmium (U-Cd; adjusted to mean specific gravity 1.012 g/mL; gestational week eight) in relation to maternal age, and stratified by parity (primiparous, 1 child, and ≥2 children).

If anything, the effect of age on the urinary cadmium concentrations appeared more pronounced among primiparous women. However, age and parity is so closely linked in the Bangladeshi women and thereby impossible to differentiate from one another.

The potential effect of pregnancy on the uptake of cadmium was further evaluated in a longitudinal analysis by measurements of cadmium in blood in gestational week 14 and six months post partum (**Paper II**). In total, the blood cadmium concentrations were about 15% higher post partum compared to early pregnancy ($p<0.001$). There could be several factors contributing to this increase in blood cadmium. First, the increased cadmium absorption likely occurred in parallel to the enhanced iron absorption, which is known to start in the second trimester and accelerate in late pregnancy, particularly in iron-deficient women (Svanberg, 1975; Bothwell, 2000). In addition, there is an increased demand of other essential micronutrients (e.g. manganese) during pregnancy (Takser *et al.*, 2004), which also may influence the intestinal absorption of cadmium by other transporters than DMT1. In animal studies, pregnancy has been shown to mobilize hepatic cadmium, leading to increased levels of cadmium and MT in blood, which then is transferred to the kidneys and placenta (Chan and Cherian, 1993). However, the relevance for humans is not known.

The increase in blood cadmium (15%) in pregnancy was about the same as that observed in a similar longitudinal study, performed on Swedish women who were more well-nourished and had lower cadmium exposure (median: 0.16 µg/L; Åkesson *et al.*, 2002) than the Bangladeshi women. The Swedish study observed a 13% increase from gestational week 11 to three months post partum. The reason for the moderate pregnancy-related increase in blood cadmium in the more malnourished Bangladeshi women may be the daily iron supplementation of 30 or 60 mg (independent of the women's iron status), which might have competed with cadmium for DMT1 binding sites as shown in experimental studies (Elisma and Jumarie, 2001). Another reason could be that blood was collected six months post partum (**Paper II**) and the half-time of cadmium in blood is two to three months. Therefore, we might have underestimated the pregnancy-related increased in blood cadmium in the Bangladeshi women. Considering that the maternal iron absorption generally decreases shortly after delivery to levels found in early pregnancy (Barrett *et al.*, 1994), a blood sample taken shortly after delivery may have shown higher blood cadmium concentrations than that six months post partum.

In order to evaluate to what extent the pregnancy-related increase in blood cadmium was influenced by the women's iron stores; women were divided into four groups depending on their iron stores (cut-off 20 µg/L) in early pregnancy and six months post partum, respectively. Women with low iron stores post partum had a higher increase in

blood cadmium (35%), compared to women with adequate iron stores (15%), independent of their iron stores in early pregnancy (**Figure 7**). Thus, the iron stores post partum seemed to be a better indicator of the women's iron status during pregnancy in the present study. Probably, those that still had low iron stores six months post partum had so low iron stores during pregnancy that the recovery of iron post delivery could not fully compensate for the losses in pregnancy and possibly during and/or shortly after delivery.

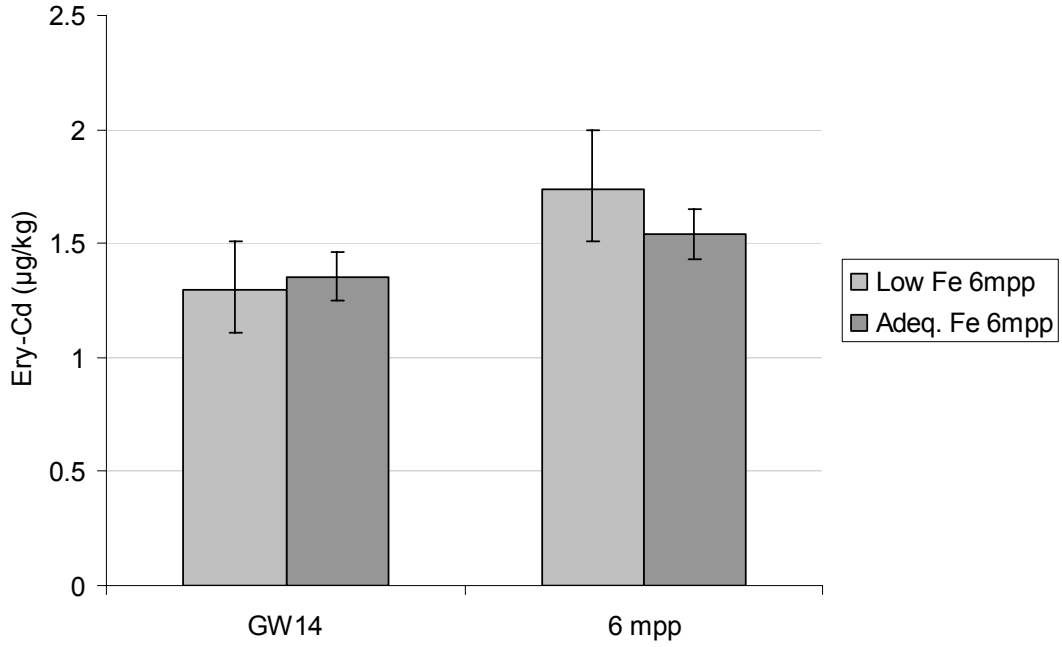

Figure 7 Geometric mean and corresponding 95% confidence intervals of cadmium in blood (erythrocyte fraction; Ery-Cd) in early pregnancy and post partum stratified by iron stores (cut-off 20 µg/L) six months post partum (6 mpp).

In conclusion, the blood cadmium concentrations increased significantly during pregnancy, especially in women with low iron stores.

4.3 PLACENTAL ACCUMULATION AND TRANSPORT

4.3.1 Cadmium in placenta and umbilical cord blood

Cadmium is known to accumulate in the placenta (Korpela *et al.*, 1986; Osman *et al.*, 2000). We found that the elevated cadmium exposure among the pregnant women in Matlab, together with the increased cadmium absorption during pregnancy resulted in high cadmium concentrations in placenta (**Paper IV**). The median cadmium concentration in placenta was 110 µg/kg dry weight, corresponding to 20 µg/kg wet

weight, with large inter-individual variations (**Table 4**). The concentrations are presented both in dry and wet weight to facilitate the comparison with other studies (**Table 4**).

In spite of the fact that the studied Bangladeshi women were non-smokers living in a non-industrialized area, the placental cadmium concentrations were much higher than in women with similar conditions, in whom the placental cadmium concentrations have been reported to be around 20 to 30 µg/kg dry weight (Korpela *et al.*, 1986; Falcon *et al.*, 2002) or 5 µg/kg wet weight (Kuhnert *et al.*, 1987; Schramel *et al.*, 1988; Osman *et al.*, 2000). Instead, the cadmium concentrations in placenta are more comparable to those reported for smokers or women living in industrially contaminated areas (Tsuchiya *et al.*, 1984; Baranowska, 1995; Odland *et al.*, 2004; Zhang *et al.*, 2004a; Sorkun *et al.*, 2007).

Table 4. The cadmium concentrations in placenta and corresponding umbilical cord blood.

	Placenta cadmium (µg/kg dry weight)	**Placenta cadmium (µg/kg wet weight)[1]**	**Umbilical cord blood cadmium (µg/kg)**
Median	110	20	0.16
Mean	130	23	0.16
Min	40	7.0	0.074
10^{th} percentile	59	10	0.12
90^{th} percentile	205	36	0.23
Max	492	86	0.32
n	44	44	41

[1]The mean ratio used for conversion from dry weight to wet weight was 5.7 (range 4.8-7.7).

The median cadmium concentration in umbilical cord blood (erythrocyte fraction) was 0.17 µg/kg (**Table Y**; **Paper IV**), which corresponds to approximately 0.1 µg/L in whole blood, assuming a hematocrit between 42 and 65% (Jopling *et al.*, 2009). There was a strong positive association between cadmium in umbilical cord blood and placenta (r_s=0.62; $p<0.001$), even though the concentration of cadmium in umbilical cord blood was less than 1% of that in placenta, which is similar to that found in a Swedish study (Osman *et al.*, 2000).

In previous studies, the cadmium concentrations in umbilical cord blood has been reported to be from 10 to 70% of the concentration found in maternal blood (Korpela *et al.*, 1986; Soong *et al.*, 1991; Lagerkvist *et al.*, 1993; Baranowska, 1995; Kantola *et al.*,

2000; Osman *et al.*, 2000). In the present study, maternal blood was not collected at delivery and therefore no direct comparison could be made with the maternal blood cadmium concentrations. However, both the cadmium concentrations in placenta (r_s=0.40; p=0.01) and umbilical cord blood (r_s=0.33; p=0.04; **Paper IV**) were positively associated with the women's urinary cadmium concentrations, despite the fact that these urine samples were collected in these women's previous pregnancy (3 to 5 years prior to the placenta sampling).

4.3.2 Impaired zinc transfer to the fetus

The zinc concentrations in umbilical cord blood decreased with increasing cadmium concentrations in placenta (r_s=-0.30; p=0.05), indicating that the accumulation of cadmum in placenta impaired the transfer of zinc to the developing fetus. This has previously been shown only *in vitro* when placentas were perfused with very high concentrations of cadmium (approximately 10000 µg/L) (Wier *et al.*, 1990), which can not be compared with the chronic exposure that the Bangladeshi women encounter. In addition, smokers had significantly lower concentrations of zinc in umbilical cord erythrocytes than non-smokers (Kuhnert *et al.*, 1987), but as tobacco smoke contains many toxic substances, besides cadmium, it is difficult to determine if the low concentration of zinc in umbilical cord erythrocytes was actually an effect of cadmium. In Swedish mother-infant pairs, no association was found between the cadmium concentrations in placenta and zinc in umbilical cord blood (Osman *et al.*, 2000). Thus, the effect in the Bangladeshi women may be related to the high prevalence of zinc deficiency (~30 to 35%; **Paper I** and **II**) and to the 4-fold higher cadmium concentrations in placenta compared to the Swedish women.

The mechanisms behind the accumulation of cadmium in placenta and the interaction with zinc are not fully understood. Several studies have suggested that MT may be involved as both metals bind to MT and induce its expression (Kuhnert *et al.*, 1987; Goyer and Cherian, 1992; Klaassen *et al.*, 1999; Ronco *et al.*, 2005; Ronco *et al.*, 2006; Sorkun *et al.*, 2007), but no clear evidence has been presented. In order to evaluate if this could be the case in the studied women, MT was first quantified in a small sub-sample of placentas (n=6; **Paper IV**). In spite of the few samples, the MT protein expression was significantly higher in the placenta samples with high cadmium concentrations (mean 370 µg/kg dry weight) than in those with low concentrations (mean 42 µg/kg dry weight; $p<0.05$). Based on these results MT was quantified in additionally 12 placentas. In the multivariate analysis, the MT protein expression was

significantly associated with the cadmium concentrations in placenta (positive; $p<0.01$), but not with zinc ($p=0.33$) or copper ($p=0.83$) in placenta. Thus, our results suggest that the accumulation of cadmium in placenta induces MT protein expression in a dose-dependent manner. However, as no association was found between MT in placenta and zinc in umbilical cord blood the results do not support the hypothesis that cadmium-induced MT mediates the impaired zinc transfer to the fetus.

Thus, the results indicate that other mechanisms except MT might be involved in the cadmium-zinc interaction in placenta. In fact, cadmium exposure was shown to down-regulate the zinc transporter ZnT-1 in conceptus in recent studies on mice (Fernandez *et al.*, 2007). Further large-scale studies on human samples are highly needed to draw any further conclusions.

In animal studies, a high cadmium exposure has been shown to interfere with the transport of several other micronutrients such as iron, copper, calcium, sodium, and potassium in the placenta (Kuriwaki *et al.*, 2005). In the studied women, the cadmium concentrations in placenta were not associated with any of the other essential micronutrients (magnesium, calcium, manganese, copper or selenium) in placenta or umbilical cord blood.

4.3.3 Potential adverse effects of prenatal cadmium exposure

The cadmium concentration in umbilical cord blood increased with increasing maternal exposure, even though a large fraction was accumulated in the placenta. In experimental studies, where dams were exposed to cadmium in environmental relevant doses, the *in utero* exposure induced earlier onset of puberty and changes in the mammary gland of female offspring (Johnson *et al.*, 2003). If the cadmium concentrations in umbilical cord blood in the present study may increase the risk of adverse health effects in the developing offspring is not known, and needs further investigation.

In the present study, the accumulation of cadmium in placental tissue impaired the transfer of zinc to the fetus. This may have serious consequences for the fetus as zinc deficiency has been associated with low birth weight, intrauterine growth retardation, and preterm delivery (Jameson, 1993). If this was the case in the studied women remains to be elucidated.

In experimental studies, perfusion of human placenta *in vitro* with different cadmium concentrations resulted in direct toxic effects such as decreased hormonal production, changed morphology of the trophoblast cells, as well as necrosis (Wier *et al.*, 1990). We are currently investigating if the accumulation of cadmium in placenta may be associated with oxidative stress and impaired immune function in the placenta. Still, the results presented above clearly emphasize the need to reduce the cadmium exposure among women in childbearing age as much as possible.

4.4 TRANSFER TO BREAST MILK

4.4.1 Cadmium in breast milk

Breast milk is the optimal infant food, providing all the nutrients needed for growth and wellbeing of the infant. Unfortunately environmental pollutants and other chemicals, to which the mother is exposed, may be transferred to milk or otherwise affect the composition of the milk.

The median concentration of cadmium in breast milk from the studied women was 0.14 µg/L (10^{th} to 90^{th} percentile: 0.063 to 0.35 µg/L; **Paper III**), which is higher than in well-nourished women in countries where rice is not the main staple food, in whom the breast milk generally contains less than 0.1 µg/L (Dabeka *et al.*, 1986; Radisch *et al.*, 1987; Hallen *et al.*, 1995; Leotsinidis *et al.*, 2005; Gundacker *et al.*, 2007; Abdulrazzaq *et al.*, 2008). On the other hand, the breast milk cadmium concentrations in the present study were in the lower range of concentrations reported from Japan and India (mean values of ~0.3-2 µg/L) (Nishijo *et al.*, 2002; Honda *et al.*, 2003a; Sharma and Pervez, 2005), considering that those studies involved colostrum, which is known to contain higher cadmium concentrations than mature milk used in **Paper III** (Gundacker *et al.*, 2007).

The cadmium concentration in breast milk increased significantly with the maternal blood cadmium concentrations (r_s=0.37; $p<0.001$; n=123; **Paper III**), and is in line with that reported for smoking women with fairly high cadmium exposure (Radisch *et al.*, 1987). Such an association was not found in previous studies with lower maternal cadmium exposure (Hallen *et al.*, 1995; Abdulrazzaq *et al.*, 2008). The reason for this is not known, but may be related to analytical difficulties at low concentrations, including higher risk of contamination.

Ideally, cadmium should have been measured also in maternal plasma as this is the cadmium fraction that is available for transport to milk. However, assuming that 5% of the whole blood cadmium is present in plasma (Karliková 2004), the measured erythrocyte cadmium concentration (median 1.5 µg/kg) would correspond to ~0.04 µg/L in plasma. Thus, the breast milk/plasma ratio for cadmium would be ~3-4, indicating no restriction in the passage of cadmium from plasma to breast milk, but rather an active transport. Therefore, the relatively low average concentration of cadmium in breast milk may be explained by the low concentration of cadmium in plasma.

The main limitation of human studies investigating cadmium transport from maternal blood circulation into the mammary gland epithelial cells and thereafter to breast milk is the availability of two compartments only, i.e. the blood and milk. Therefore, no conclusions can be made about the accumulation of cadmium within the mammary gland, as shown in experimental studies (Bhattacharyya *et al.*, 1982; Petersson Grawé and Oskarsson, 2000).

4.4.2 Interactions with micronutrients in breast milk

The present study is the first to detect strong positive associations between cadmium and both iron (r_s=0.55; p<0.01) and manganese (r_s=0.56; p<0.01) in breast milk (**Paper III**). The associations suggest common transporters or pathways in the mammary gland, as previously indicated for intestinal uptake (**Paper II)**. The key candidate in the common intestinal uptake is DMT1, which is known to transport all three metals (Gunshin *et al.*, 1997). In plasma, iron is mainly bound to transferrin (Tf) and uptake into mammary epithelial cells is mediated by the cellular transferrin receptor (TfR). Once Tf binds to TfR it is internalized and fused with acidic endosomes where iron is released and possibly exported to the cytoplasm by DMT1 (Leong and Lönnerdal, 2005; Lönnerdal, 2007). FPN1, on the other hand, is localized throughout the mammary epithelial cell and is believed to transport iron into the secretory vesicles targeted for export to milk (Kelleher and Lönnerdal, 2005; Leong and Lönnerdal, 2005; Lönnerdal, 2007). Very little has been reported about manganese transport in the mammary gland, but it is likely to involve binding to Tf, uptake by the TfR, and subsequent transport by DMT1, like iron (Gunshin et al. 1997; Scheuhammer and Cherian 1985). Probably, cadmium is transported in a similar manner as it also may bind to Tf (Harris and Madsen, 1988). The simultaneous increase of these three metals in breast milk is likely due to the fact that Tf is not fully saturated with iron (Harris and

Madsen, 1988), which possibly allows both manganese and cadmium to bind to Tf without displacement of iron and be taken up via TfR.

Iron as well as a large part of the manganese in human milk is bound to lactoferrin (Lönnerdal *et al.*, 1985; Lönnerdal and Iyer, 1995), but it is not known if cadmium binds to lactoferrin. It has just been reported that cadmium is not statistically associated with lactoferrin (Mata *et al.*, 1996; Milnerowicz *et al.*, 2001).

Interestingly, there was a negative association between cadmium and calcium in breast milk (r_s=-0.17; $p \leq 0.05$; **Paper III),** similar to that found in blood (**Paper II**). Calcium in breast milk was not influenced by any of the demographic factors, but was positively associated with magnesium, copper and selenium in breast milk. However, the negative association between cadmium and calcium remained significant (Beta=-0.27; $p \leq 0.01$) after adjusting for these other micronutrients. It is remarkable that even though the cadmium concentration was more than six orders of magnitude lower than that of calcium, it appeared to inhibit the secretion of calcium to breast milk. Honda and coworkers have previously reported a similar negative association in colostrum of 68 Japanese women (Honda *et al.*, 2003a). In mice, cadmium exposure reduced the expression of the milk protein β-casein, the synthesis of which is calcium-dependent, and high cadmium doses decreased the calcium concentration in the mammary gland (Öhrvik *et al.*, 2006). The mechanism behind the negative association between cadmium on calcium is not clear, but it is known that even low concentrations of cadmium can disturb the tightly regulated concentrations of intracellular calcium either by blocking calcium-channels and/or by binding to calcium transporters (Bridges and Zalups, 2005). The average calcium concentration in the mature breast milk collected from the studied women was 260 mg/kg (**Paper III**), which is at the lower end of the reported normal range of 250 – 340 mg/L (Lönnerdal, 1997; Lönnerdal, 2000). In Matlab, the dietary intake of calcium is low (Kimmons et al. 2005), but generally this is not believed to affect the calcium concentration in breast milk (Lönnerdal 1997). However, it could render the women more sensitive to factors affecting the calcium concentration, such as cadmium.

4.4.3 Potential adverse effects of postnatal cadmium exposure

The maternal cadmium exposure influenced the concentration of cadmium in breast milk. Although the concentrations in breast milk seem low, the higher gastrointestinal uptake in the infant (Crews *et al.*, 2000) may lead to a higher cadmium uptake per kg

body weight than in the mother. Thus, it may contribute significantly to the body burden of the infant, especially since the half-time of cadmium in the body is in the order of decades. In addition, experimental studies have shown that cadmium exposure via milk may cause neurotoxicity in the offspring (Andersson *et al.*, 1997; Petersson Grawé *et al.*, 2004), but the relevance to humans remains to be elucidated.
The present results indicate that cadmium inhibits the secretion of calcium to breast milk, possibly leading to decreased calcium intake in the infant. Assuming a linear relationship between cadmium and calcium in breast milk, a cadmium concentration of 0.5 µg/kg was associated with approximately 15% decrease in calcium. The consequences of this is not known, but considering the low concentrations of calcium in breast milk of the studied women a further decrease due to cadmium exposure is obviously not a promising finding. We have initiated studies on adverse effects of cadmium on bone development in the present cohort.

In spite of the negative findings presented above it is important that women are encouraged to breast-feed, as breast milk is an important source of balanced nutrition and passive immunization for the infant. Indeed, it plays a central role in the child's development. It should also be noted, that milk-based infant formulas are expensive in Bangladesh and therefore poor families are often forced to give their infants complementary food based on rice, wheat or barley (Das *et al.*, 1992), which are likely to contain much more cadmium than breast milk (Eklund and Oskarsson, 1999). Therefore, we emphasize the need to reduce the cadmium exposure as much as possible. Also, further research on the consequences of the early life cadmium exposure is highly warranted.

5 CONCLUSIONS

- ✓ Women in Matlab, Bangladesh, have elevated cadmium exposure, probably mainly via rice.

- ✓ The urinary cadmium concentrations were similar to those that have been associated with increased risk of adverse health effects on kidneys and bone.

- ✓ The intestinal uptake of cadmium appears to be mediated via iron/manganese transporters (e.g. DMT1), especially in women with adequate zinc status, but not to any significant extent via zinc transporters.

- ✓ The intestinal uptake of cadmium is increased during pregnancy, especially in women with low iron stores.

- ✓ Cadmium accumulates in the human placenta and thereby impairs the transfer of zinc to the fetus, possibly via other mechanisms than binding to MT.

- ✓ Cadmium appears to disturb calcium transport both in the intestine and in the mammary gland, possibly by blocking calcium channels and/or by binding to calcium transporters.

- ✓ The transfer of cadmium to breast milk was low, but seemed to be mediated via the active transport system utilized by iron and manganese.

6 FUTURE RESEARCH

The most important task in relation to environmental cadmium exposure is to find means to reduce the dispersion of cadmium in the environment and thereby reduce the exposure. In industrialized countries, cadmium exposure and the related adverse health effects have received much attention, which has lead to strict regulations and monitoring of emissions of cadmium to the environment. However, in a recent report by UNEP (UNEP, 2006) it was emphasized that there is a need of more data on the release of cadmium in developing countries. The present research has contributed to some increased knowledge on the cadmium exposure in rural Bangladesh. Furthermore, it has increased the understanding of how cadmium may interact with different essential micronutrients for uptake and transport in humans, but there are of course many areas that need further research. For example:

- ✓ Further studies assessing cadmium exposure and the related adverse health effects in Bangladesh, especially in women.

- ✓ Studies that measure the cadmium concentration in different foods that are frequently consumed in Bangladesh, such as various types of rice.

- ✓ To investigate if the pre- and postnatal cadmium exposure that emerges from environmental relevant doses in humans may be associated with adverse health effects in the offspring.

- ✓ Assessment of the consequences of impaired zinc transport to the fetus, as well as impaired calcium transport to milk for the developing child.

- ✓ Further large-scale studies that elucidate the mechanism behind the interaction between cadmium and zinc in human placenta.

- ✓ Mechanistic studies that investigate how cadmium disturbs the transport of calcium in the intestine and in the mammary gland.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kadmium är ett metalliskt grundämne som inte har någon biologisk betydelse för människor eller djur och den kan inte heller brytas ner. Denna metall upptäcktes redan 1817 men det var först i början av 1900-talet som kadmium började användas flitigt inom industrin (t.ex. stabilisator i plast, färgpigment, legeringar, korrosionsskydd, nickel-kadmium batterier). Utsläpp av kadmium till luft sker främst av sopförbränning, vid metalltillverkning och av förbränning av fossila bränslen. Kadmium finns naturligt i alla jordar men det tillförs även till våra åkermarker via luftföreningar, handelsgödsel, rötslam och i viss mån via stallgödsel.

Kadmium finns i alla livsmedel, men vanligtvis är halterna låga. Största problemet med kadmium är att det lätt tas upp av växternas rotsystem. Den största exponeringskällan är därför baslivsmedel, såsom spannmålsprodukter (främst vete och ris), potatis, rotfrukter och grönsaker. Rökning ger också en betydande exponering, eftersom tobak ofta innehåller mycket kadmium och upptaget från lungorna till blodet är högt. Vanligtvis är upptaget av kadmium från tarmen till blodet betydligt lägre (<5% av intaget), men kan variera beroende på typ av föda och individens nutritionsstatus. Upptaget av kadmium ökar vid låga järndepåer, vilket troligen beror på att kadmium kan tas upp via samma transportör som tar upp järn i tarmen. Flera djurstudier har visat att kadmium även kan utnyttja transportsystemen för ett flertal andra livsnödvändiga näringsämnen, men om detta även sker hos människor är inte känt.

Det kadmium som tas upp i kroppen ansamlas framförallt i njurarna och kroppen saknar en bra förmåga att göra sig av med kadmium via urin och avföring. Denna ansamling av kadmium i kroppen leder med tiden till ett flertal negativa hälsoeffekter, främst på njurarna. Den livslånga exponeringen leder även till risk för effekter på ben och kroppens hormonella system.

Detta projekt syftar till att öka kunskapen om kadmiumexponeringen hos gravida kvinnor och deras barn på landsbygden i Bangladesh. Dessa kvinnor är troligen en riskgrupp för hög kadmiumexponering då många är undernärda vilket kan leda till ökat upptag från födan samt att deras huvudsakliga basföda består av ris som man vet kan innehålla höga halter av kadmium. Ett annat syfte med dessa studier var att utreda hur kadmium kan störa transporten av livsnödvändiga näringsämnen däribland zink och kalcium i kroppen.

Resultaten i denna avhandling visar att kvinnor på landsbygden i Bangladesh är exponerade för relativt höga kadmiumhalter. Kadmiumhalterna i urinen hos de undersökta unga kvinnorna i Bangladesh var nästan lika höga som hos medelålders kvinnor i Sverige, vilka uppvisar tecken på effekter på både njurar och ben till följd av den livslånga kadmiumexponeringen via födan. Kvinnor med järnbrist hade klart högre kadmiumhalter i både blod och urin än kvinnor med bra järnvärden, framförallt de kvinnorna som hade bra zinkstatus. Detta kan bero på att zink är viktig för regleringen av järnupptaget i kroppen. Ett helt nytt fynd är att kadmium samverkar med den livsnödvändiga metallen mangan, vilket kan bero på att även upptaget av mangan ökar vid låga järndepåer. Ett annat viktigt fynd är att kadmium verkar minska upptaget av kalcium i tarmen troligen genom att blockera en del av de transportörer som tar upp kalcium.

Kadmiumhalterna i blod ökade under graviditeten, speciellt bland kvinnor med järnbrist. Vi tror att detta är en följd av det ökade järnbehovet under graviditeten, som ökar upptaget av både järn och kadmium från födan. Genom att analysera moderkakan och tillhörande navelsträngsblod kunde vi visa att mycket kadmium fastnar i moderkakan och endast mindre mängder går över till fostrets blod. Ansamlingen av kadmium i moderkakan verkar dock leda till en minskad transport av zink till fostret. Detta kan ge allvarliga följder, eftersom zink behövs för fostrets tillväxt och utveckling. Kadmiumhalterna i bröstmjölk var tämligen låga men ökade med moderns kadmiumexponering. Resultaten tyder på att kadmium utnyttjar samma transportsystem i mjölkkörteln som i tarmen d.v.s. de som transporterar de livsnödvändiga metallerna järn och mangan. Vi kunde även visa att kadmium minskar halten av kalcium i bröstmjölk, möjligen genom att blockera de transportörer som utsöndrar kalcium till bröstmjölk. Om de låga kadmiumhalter som överförs till fostret via moderkakan och efter födseln via bröstmjölk påverkar barnets utvecklig är ännu inte känt och det krävs mer forskning. Sammanfattningsvis så är den viktigaste uppgiften i framtiden att på alla sätt minska spridningen av kadmium i miljön och därigenom även exponeringen.

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