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Genetic variability and cadmium metabolism and toxicity

Rentschler, Gerda

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GENETIC VARIABILITY AND CADMIUM METABOLISM AND TOXICITY

Gerda Rentschler

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Abstract: Cadmium (Cd) is ubiquitous in the environment. Human exposure in non-smokers occurs mainly via intake of healthy food like vegetables, cereals, and shellfish. Adverse health effects on kidney and bone at low-level environmental Cd exposure are well-documented in adults. There is considerable inter-individual variation in both metabolism (toxicokinetics) and toxicity (toxicodynamics) of Cd. This may be due to genetic factors.

The aim of this thesis was to identify genetic factors that are associated with Cd metabolism and toxicity.

As Cd is a foreign element, there is no endogenous metabolism of uptake. However, there is evidence that the transporters of other elements such as iron and zinc are not sufficiently specific to prevent uptake of Cd. Further, the zinc-binding protein metallothionein (MT) protects against toxicity of Cd in the kidney. Thus, the first study examined the association of single nucleotide polymorphisms (SNPs) in iron homeostasis genes with Cd concentrations in two groups of women, one from the Argentinean Andes and the other from rural Bangladesh. The second study examined the association of SNPs in two zinc homeostasis genes, *SLC39A8* and *SLC39A14* with Cd concentrations in the same groups of women as in the first study. The third study analysed whether polymorphisms in the metallothionein genes *MT1A* and *MT2A* influence Cd-related kidney damage in volunteers from three areas in southern China with various degrees of Cd pollution. All study designs were cross-sectional.

We found (1) that one SNP in the iron-related transferrin receptor gene *TFRC* (rs3804141) was associated with Cd concentrations in urine in both women from Argentina and Bangladesh: carriers of AA had 56-58% and carriers of GA had 22% higher urinary Cd concentrations than GG. The consistency of the results in two different populations hints at a causal relation. Further, we found (2) that SNPs in the zinc-related genes *SLC39A14* and *SLC39A8* were associated with blood Cd concentrations, for *SLC39A14* this may occur via differential gene expression. Finally, we found (3) that AA carriers of rs11076161 *MT1A* had higher Cd concentrations in blood among individuals in the highest Cd exposure group. Also, with increasing Cd exposure, carriers of this genotype experienced more Cd-related kidney toxicity.

The present data are of significance for the theoretic understanding of the metabolism and toxicology of Cd, and, at the same time they are valuable in risk assessment, an issue of great importance, in light of the non-existing range between present exposure to Cd and toxicity in many parts of the world, including Sweden.

Key words Cadmium, polymorphism, *TFRC, SLC39A8, SLC39A14, MT1A*

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GENETIC VARIABILITY AND CADMIUM METABOLISM AND TOXICITY

Gerda Rentschler

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CONTENTS

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POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Kadmium är en giftig metall vilken finns i mat som vi rekommenderas äta mycket av, exempelvis sädesprodukter, grönsaker och skaldjur. Våra matjordar är kontaminerade med kadmium och föroreningen kommer att kvarstå under mycket lång tid framöver. Kadmium skadar framförallt njurarna och skelettet och kan antagligen även orsaka vissa typer av cancer. Människor är olika känsliga för toxiska effekter av kadmium, och resultat tyder på en ärftlig orsak till denna skillnad i känslighet, men bakgrunden är oklar.

Detta arbete vill identifiera ärftliga faktorer hos människa vilka påverkar upptag och omsättning av kadmium i kroppen samt dess toxicitet.

Kadmium är ett främmande ämne och därför finns det inga speciella gener eller proteiner som reglerar dess omsättninger. Man vet att proteiner som omsätter de essentiella näringsämnena zink och järn inte är hel specifika, och därmed också kan påverka omsättningen av kadmium. Hos kvinnor med lågt järnvärde, vilket kan förekomma t.ex. under graviditet, har det uppmätts förhöjda kadmiumkoncentrationer. Dessutom vet man att en grupp av proteiner som binder zink, så kallade metallothioneiner, skyddar njurarna från kadmiums giftighet.

Syftet med den första studien var att leta efter samband mellan variationer i järnrelaterade gener och kadmiumkoncentrationer i blod och urin hos två grupper av kvinnor. Den ena var från Bangladesh och den andra från argentinska Anderna. Syftet med den andra studien var att leta efter samband mellan gener som tillhör zinkmetabolismen och kadmiumkoncentrationer i samma två grupper. Syftet med den tredje studien var att hitta samband mellan variationer i metallothionein-gener och kadmiumkoncentrationer i blod och urin samt njurskada i en grupp män och kvinnor från södra Kina.

En variation i transferrinreceptor-genen visade sig vara förknippad med högre kadmiumkoncentrationer i urin hos båda kvinnogrupperna, särskilt bärare av den mer ovanliga varianten (ca 26% av kvinnorna i Anderna och 14% i Bangladesh). Dessutom fanns det samband mellan variationer i zinkrelaterade gener och kadmiumkoncentrationer i blod hos kvinnorna från Anderna. Kinastudien visade att bärare av en polymorfi i metallothionein 1A-genen hade högre risk för njurskada vid stigande exponering för kadmium.

Mycket lite har hittills varit känt om vilka gener som styr omsättningen och toxicitet av kadmium i människokroppen. Detta arbete har bidragit till kunskap om vilka gener, och mer specifikt vilka genvarianter, som har betydelse för kadmiums anrikning i människan och om dessa påverkar kadmiums skadlighet.

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LIST OF PAPERS
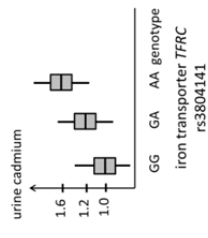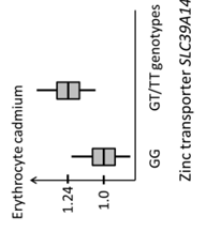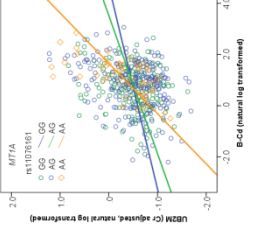
This thesis is based on the following papers, which are appended at the end.

I. Polymorphisms in iron homeostasis genes and urinary cadmium
    concentrations among nonsmoking women in Argentina and Bangladesh.
    Rentschler G, Kippler M, Axmon A, Raqib R, Ekström EC, Skerfving S,
    Vahter M, Broberg K.
    Environ Health Perspect. 2013 Apr;121(4):467-72, 472e1-7. doi:
    10.1289/ehp.1205672. Epub 2013 Feb 14.

II. Cadmium Concentrations in Human Blood and Urine are Associated with
     Polymorphisms in Zinc Transporter Genes.
     Gerda Rentschler, Maria Kippler, Anna Axmon, Rubhana Raqib, Staffan
     Skerfving, Marie Vahter, Karin Broberg.
     Accepted Metallomics, DOI:10.1039/C3MT00365E

III. A polymorphism in metallothionein 1A (*MT1A*) is associated with
      cadmium-related excretion of urinary beta 2-microglobulin.
      Lei L, Chang X, Rentschler G, Tian L, Zhu G, Chen X, Jin T, Broberg K.
      Toxicol Appl Pharmacol. 2012 Dec 15;265(3):373-9. doi:
      10.1016/j.taap.2012.09.006. Epub 2012 Sep 17

ABBREVIATIONS

B-Cd	Blood cadmium concentration
B2M	Beta-2-microglobulin
BMI	Body Mass Index
Cd	Cadmium
CI	95% confidence interval
Ery-Cd	Erythrocyte cadmium concentration
FDR	False Discovery Rate
LD	Linkage Disequilibrium
MT	Metallothionein
NAG	N-acetyl-beta-D-glucosaminidase
rs	Reference SNP ID
r_S	Spearman´s rank correlation coefficient
SNP	Single nucleotide polymorphism
SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (DMT1)
SLC39A8	Solute carrier family 39 (zinc transporter), member 8, (alternative name ZIP8)
SLC39A14	Solute carrier family 39 (zinc transporter), member 14, (alternative name ZIP14)
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1 (ferroportin 1)
TF	Transferrin
TFR2	Transferrin receptor 2
TFRC	Transferrin receptor (p90, CD71)
U-Cd	Urinary cadmium concentration
UNAG	Urinary N-acetyl-beta-D-glucosaminidase concentration
UB2M	Urinary beta-2-microglobulin concentration

THESIS AT A GLANCE

Paper	Objective	Population	Study design	N	Outcome marker	Explanatory variables	Main result
Paper I	SNPs in iron related genes ⤢ Cadmium metabolism	Bangladesh Argentina	Cross-sectional	359 women 172 women	Blood and urinary cadmium	Genotypes, Age, Plasma ferritin, BMI, Gene expression	urine cadmium; GG GA AA genotype iron transporter *TFRC* rs3804141
Paper II	SNPs in SNPs in zinc-related genes *SLC39A8* and *SLC39A14* ⤢ Cadmium metabolism	Bangladesh Argentina	Cross-sectional	400 women 172 women	Blood and urinary cadmium	Genotypes, Age, Plasma ferritin, BMI Parity, Plasma zinc, Gene expression	Erythrocyte cadmium; GG GT/TT genotypes Zinc transporter *SLC39A14*
Paper III	SNPs in metallothionein *MT1A* and *MT2A* ⤢ Cadmium metabolism Cadmium toxicity	China	Cross-sectional	512 men and women	Blood and urinary cadmium UB2M UNAG	Genotypes, Age, Sex, Smoking	

INTRODUCTION

Gene-metal interactions

There is a wide inter-individual variation as regards effects of exposure to toxic agents. Such variation may have many reasons and some may be because of variation in genetic traits. The genetics may influence both the toxicokinetics (metabolism, i.e. absorption, distribution, biotransformation, or elimination) and toxicodynamics (toxic effects). This means that the shape of the dose-response curve is affected, i.e. a certain exposure will result in inter-individual differences in concentrations of the agents (or its metabolites) in biomonitoring media and/or toxic effects. It will also mean that populations with differences in genetic traits will react differently at a certain level of exposure.

Metals are interesting from a toxicological point of view, since exposures above or close to toxic levels are often present in occupational and general environments. Also, there is a wide variation in metal toxicity between individuals (Dickens 1868). Thus, metals are also interesting models for gene-environment interactions in humans. Further, it is often possible to find populations with well-defined exposures, and biomonitoring offers the possibility to study interactions as regards metabolism and dose-response relationships. Some individuals accumulate metals in the body over time, and, therefore, even a moderate difference in toxicology and a minor difference in frequency of a genotype can make a significant difference for risk assessment.

Hence, metals have been assessed for gene-environment interactions in the last decade. The most studied one in humans is lead. It has been shown that polymorphisms in the gene encoding delta-aminolevulinic acid dehydratase (*ALAD*) influence both toxicokinetics (Tian et al. 2013; Zheng et al. 2011) and toxicodynamics (Pawlas et al. 2012; Tian et al. 2013) of lead. Interestingly, there is a wide variation in genotype frequencies between populations (Pawlas et al. 2012; Tian et al. 2013; Zheng et al. 2011). Also, a polymorphism in the vitamin D receptor gene (*VDR*) is associated with differences in both toxicokinetics (Garcia-Leston et al. 2012) and toxicodynamics (Pawlas et al. in press) of lead.

Another example is arsenic where there is pronounced interaction between polymorphisms and exposure, both for toxicokinetics (Engström et al. 2011; Schläwicke Engström et al. 2009) and toxicodynamics (Engström 2010). Again, the genotype frequencies vary globally and there may even be genetic selection in

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populations with high exposure during long time (Schlebusch et al. 2013). The toxicokinetics of mercury, both inorganic (Engström et al. 2013; Harari et al. 2013) and methylmercury (Schläwicke Engström et al. 2008) vary according to genetics. There is also gene-environment interaction for manganese (Rentschler et al. 2012). Beryllium is another metal, the toxicology of which is affected in a clinically significant way (Richeldi et al. 1993). Further, there are indications that the metabolism of nickel is influenced by genetic traits (Ross-Hansen et al. 2013).

In spite of toxicologically significant exposure to cadmium (Cd) in many populations, and inter-individual variation in susceptibility in humans, there is little information on potential impact of genetics on Cd metabolism and toxicity.

There are different ways for genetic variants to interact with metal toxicity (Nordberg et al. 2007a). The majority of the studies cited above studied gene-metal interactions as a result of single-nucleotide polymorphisms (SNPs; Figure 1). SNPs can be non-synonymous, which will result in a different protein and, thus, its association with metal exposure, as is the case e.g. for lead (Zheng et al. 2011) and beryllium (Richeldi et al. 1993). On the other hand, intronic SNPs can also be associated with the phenotype, e.g. by controlling transcription, which will affect the amount of protein produced and thus, the interaction with the metal, as is the case for arsenic (Engström et al. 2011). However, there are other mechanisms for SNPs to be associated with toxicokinetics or toxicodynamics, e.g. the studied SNP might be in linkage disequilibrium (i.e. inherited together) with the functional SNP. In this thesis on gene-environment interaction for Cd, only non-synonymous and intronic SNPs are studied (Figure 1).
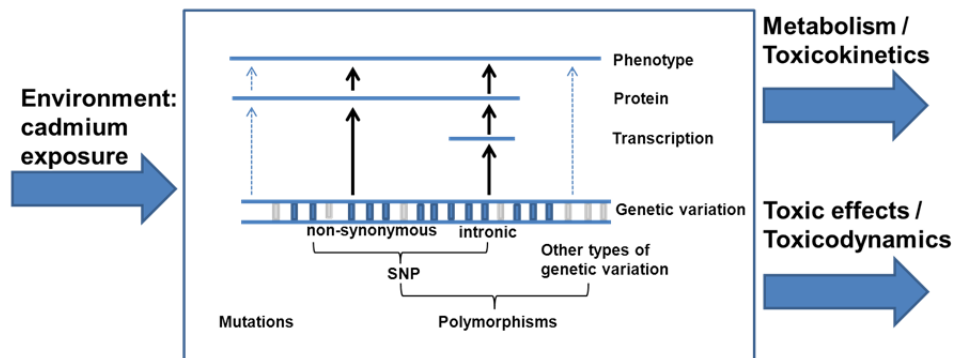

Figure 1: Schematic of different possibilities of genetic variations to interact with environmental cadmium exposure in relation to toxicokinetics or toxicodynamics. Bold back arrows: studied in this thesis.

Cadmium

Cd is ubiquitous in the environment. Human exposure occurs via plant-derived foods, and certain seafoods (Olsson et al. 2002), as well as from tobacco smoke (Bensryd et al. 1994; Olsson et al. 2002). Adverse health effects on kidney (Åkesson et al. 2005) and bone (Åkesson et al. 2006; Engström et al. 2012) at low-level environmental Cd exposure are well-documented in adults. Also, recent studies have reported increased risk of hormone-related cancers with increasing Cd in food (Åkesson et al. 2008; Julin et al. 2012).

Cd is absorbed from the gastrointestinal tract (Nordberg et al. 2007b; Figures 2 and 3). In blood plasma, it is bound to albumin and transported to the liver, where it is bound to the zinc-containing low molecular weight protein metallothionein (MT), which is transported in blood plasma to the kidney, filtered through the glomeruli and reabsorbed in proximal tubuli, where it accumulates (Nordberg et al. 2007b). MT protects the kidney by its binding of Cd; toxic effects occur only when the capacity of MT synthesis is exceeded.

The first sign of kidney toxicity is increased urinary excretion of low molecular weight proteins, such as beta-2-microglobulin (UB2M), reflecting effects on the proximal tubuli with decreased reabsorption of low molecular weight proteins and of N-acetyl-beta-D-glucosaminidase (UNAG), which reflects shedding of tubular epithelium (Åkesson et al. 2005; Jin et al. 1999; Liang et al. 2012)

The Cd concentration in blood (B-Cd) is used as a biomarker of mainly recent exposure. In the blood, almost all Cd is present in the erythrocytes, the level in which may be used for monitoring (Ery-Cd). The turnover of Cd in the body is very slow (half-time decades). Hence, there is accumulation in the body, to a large part in the kidney. Urinary Cd concentration (U-Cd) is an index of the body burden.

There is considerable inter-individual variation in both metabolism (toxicokinetics) and toxicity (toxicodynamics) of Cd. This may be due to genetic factors being associated with Cd metabolism (Björkman et al. 2000) and toxicity (Nordberg et al. 2007b). Genetic interaction with Cd metabolism may be reflected by changes of B-Cd and U-Cd. Genetic variation in in the protective MT may interact with toxic effects on the proximal tubuli. However, there is need for further and more specific information.

Gene-environment interactions are most likely of importance for the risk assessment of Cd. Both biomarkers of Cd metabolism (B-Cd and U-Cd) are extensively used for assessment of exposure, and retention and inter-individual

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variation in Cd-metabolism will affect their usability. Further, variation in toxicodynamics will affect the shape of the dose-response curve for urinary excretion of low molecular weight proteins, which is crucial in risk-assessment (EFSA 2009, 2011; JECFA 2010).

As Cd is a non-essential element, there is no specific gastrointestinal uptake. However, there is evidence that the transporters of other elements, such as iron and zinc are not sufficiently specific to hinder uptake of Cd. Also, there is evidence from animal studies that zinc metabolism interacts with that of Cd (Nordberg et al. 2007b).

Interaction with iron metabolism

Especially women and adolescent girls are prone to have low iron stores. In correlation with decrease of iron stores (measured as plasma ferritin), Cd
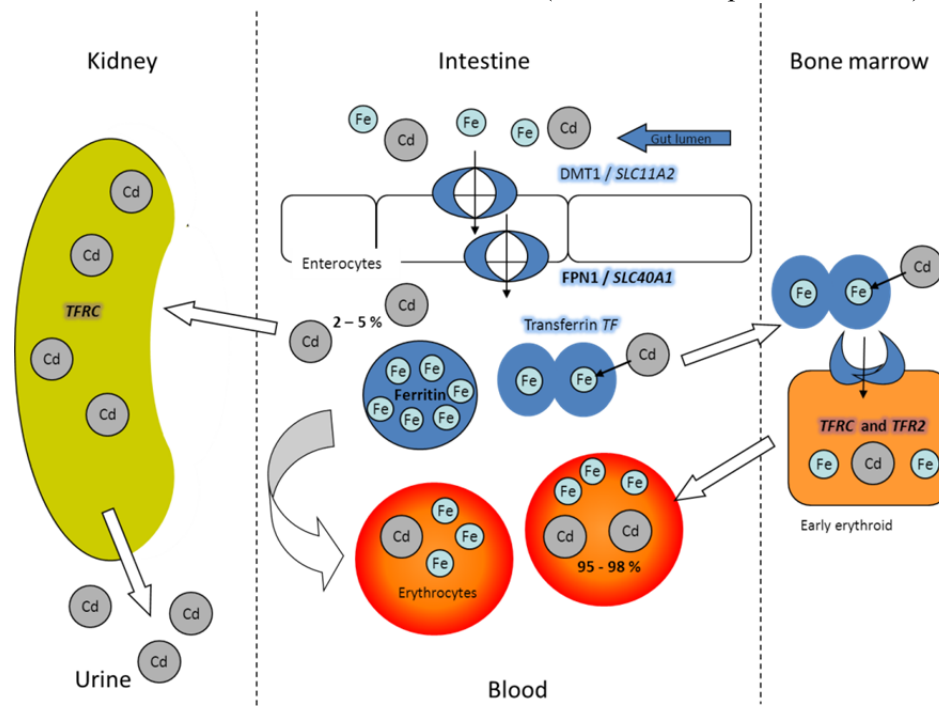
Figure 2: Schematic of possible interactions of Cd with the iron metabolism: in the intestine, Cd is probably transported into cells by DMT1 and exported by FPN1. In blood, 95-98% of Cd is bound to erythrocytes and 2-5% to plasma proteins, among them transferrin. Iron bound to transferrin is imported into cells (e.g. early erythroids) by transferrin receptors TFRC and TFR2. This might be one of several possible entryways of Cd into erythrocytes. In the kidney Cd is filtered in glomeruli and most of it is re-absorbed in proximal tubuli. Only a minor amount is released in urine.

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concentrations are increased in blood or urine (Bárány et al. 2005; Kippler et al. 2007; Meltzer et al. 2010; Skerfving et al. 1999). The most well-studied interaction of Cd with the iron metabolism is for the divalent metal transporter 1 (DMT1, gene name *SLC11A2*; Bressler et al. 2004; Tallkvist et al. 2001) which is upregulated in the intestine in situations of low iron status, to increase the uptake of iron and, as a side effect, probably more Cd will be taken up (Figure 2). However, other iron-related genes are also associated with Cd; mobile transferrin has been shown to bind Cd instead of iron in solution (Harris and Madsen 1988) and ferroportin, the product of *SLC40A1* can export Cd and other metals from cells and its expression is increased with increasing Cd concentrations (Park and Chung 2009; Troadec et al. 2010). The transferrin receptors (*TFRC* and *TFR2*) play a major role in iron homeostasis. They act in concert in all cells that need iron but are most highly expressed in early erythroids to import transferrin and release the iron within the cell (Wang and Pantopoulos 2011).

Interaction with zinc metabolism

There is evidence from animal studies that zinc-homeostasis genes interact with Cd metabolism (Nordberg et al. 2007b). In cell cultures and animal experiments, expression of other zinc-homeostasis genes, mainly *SLC39A8* and *SLC39A14,* have been linked with Cd uptake or resistance (Fujishiro et al. 2012; Girijashanker et al. 2008; Wang et al. 2007). A schematic of possible interaction of Cd with zinc homeostasis is given in Figure 3.

As said above, the zinc-binding protein MT protects against the toxicity of Cd (Nordberg et al. 2007b). Genes in the MT family have long been known to affect Cd metabolism. The genes of this family are located on chromosome 16 in a highly repetitive region. Therefore, it has been difficult to genotype SNPs unambiguously. However, there has been some evidence linking mainly one SNP in *MT2A* with modified Cd concentrations in humans (rs28366003 (Kayaalti et al. 2011; Kayaalti et al. 2010; Tekin et al. 2011). Other SNPs in *MT1A* (rs11076161) and *MT2A* (rs10636) have also been associated with aging, diabetes and atherosclerosis (Giacconi et al. 2007; Mazzatti et al. 2008; Mocchegiani et al. 2008; Yang et al. 2008). The disease-associations of these SNPs are possibly through association with zinc transport, which makes them candidates to study whether they are also associated with Cd transport.

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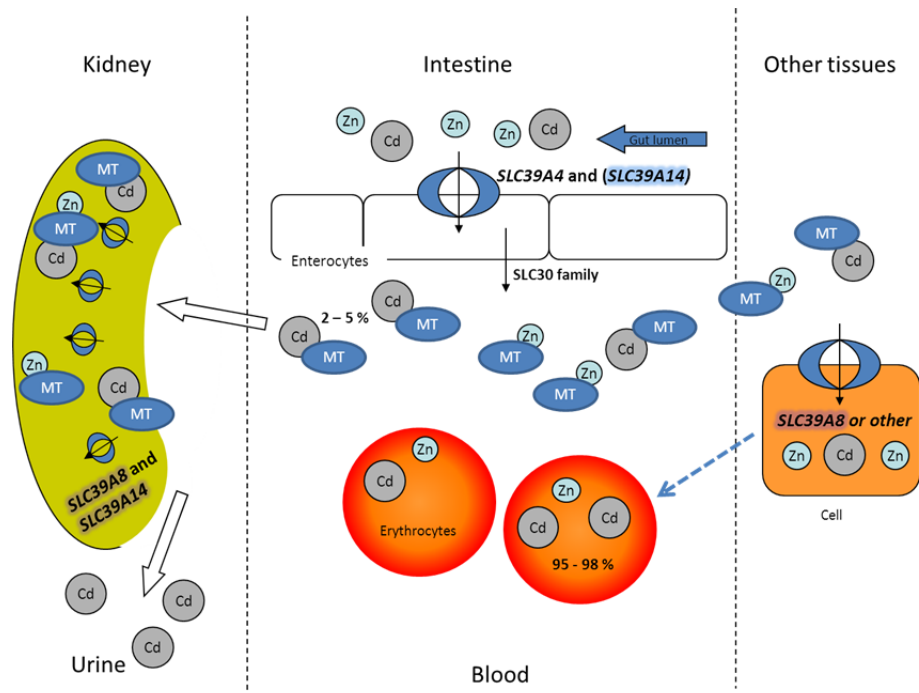
Figure 3: Schematic of possible interaction of Cd with the zinc homeostasis: Cd could in the intestine be transported into enterocytes via zinc-transport proteins, such as SLC39A14 and exported to blood stream by transporters of the SLC30 family. In blood, 95-98% of Cd is bound to erythrocytes and 2-5% to plasma proteins, among them metallothioneins (MT). Zinc is imported into cells via transporters of the SLC39 family, among them SLC39A8 and this could also be a possible entryway for Cd. In the kidney, Cd bound to MT is filtered in glomeruli and most of it is re-absorbed in proximal tubuli. Only a minor amount is released in urine.

AIMS

Overall aims

Cadmium toxicokinetics and toxicodynamics may be modified by genetic factors. However, the knowledge is very limited. Therefore, the general aim of this thesis is to gain better understanding of genetic influences on Cd toxicokinetics and toxicodynamics.

Specific aims

- To elucidate whether variation in iron and zinc genes is associated with Cd metabolism.
- Specifically, to find out whether sequence variations in the *TFRC, SLC11A2, SLC40A1, TFR2,* and *TF* genes are associated with Cd metabolism.
- To survey whether variation in *TFRC, SLC11A2, SLC40A1, TFR2,* and *TF* influences Cd metabolism by modification of gene expression.
- To investigate whether sequence variations in the zinc genes *SLC39A8* and *SLC39A14* are associated with Cd metabolism.
- To survey whether variation in *SLC39A8* and *SLC39A14* influences Cd metabolism by modification of gene expression.
- To elucidate whether sequence variation in metallothionein genes *MT1A* and *MT2A* is associated with Cd metabolism and toxicity.

MATERIALS AND METHODS

Populations

In the three papers, three different population groups were included. Paper I was based on samples from 172 women from the Argentinean Andes and 359 women from Bangladesh. Paper II was based on the same women from Argentinean Andes and 403 women from Bangladesh. Paper III comprised samples from 512 men and women from southern China who were exposed to different degrees of Cd pollution.

Table 1. Summary of the study populations and analyses.

Paper	Study population	Year of sampling	Cd bio-markers[a]	Effekt markers[b]	Other variables[c]	Genes studied
I	Argentinean Andes	2008	B-Cd, U-Cd		Age, BMI, P-ferritin, Gene expression	*TFRC, TFR2 SLC11A2, TF, SLC40A1*
	Bangladesh	2002	Ery-Cd, U-Cd		Age, BMI, P-ferritin, Gene expression	*TFRC, TFR2 SLC11A2, TF, SLC40A1*
II	Argentinean Andes	2008	B-Cd, U-Cd		Age, BMI, Parity, P-ferritin, P-Zn, Gene expression	*SLC39A8, SLC39A14*
	Bangladesh	2002	Ery-Cd, U-Cd		Age, BMI, Parity, P-ferritin, P-Zn, Gene expression	*SLC39A8, SLC39A14*
III	China	2006	B-Cd, U-Cd	UNAG, UB2M	Age, Sex, Smoking	*MT1A, MT2A*

[a] B-Cd = Blood cadmium concentration; U-Cd = Urinary cadmium concentration; Ery-Cd = Erythrocyte cadmium concentration;
[b] UNAG = Urinary N-acetyl-beta-D-glucosaminidase; UB2M = Urinary beta-2-microglobulin
[c] BMI = Body mass index; P-ferritin = Plasma ferritin concentration; P-Zn = Plasma zinc concentration;

The study populations from Argentina and Bangladesh were originally recruited to study the health effects of arsenic in drinking water. Because the individuals also are exposed to Cd to different degrees, these populations were chosen to elucidate gene-environment interactions for Cd. Therefore, for Papers I-II it was not necessary to perform any new sampling and only stored samples were used. An overview of the study groups, genes and variables studied is given in Table 1. All study designs were cross-sectional. In Paper I genetic variation of genes from the iron homeostasis system was studied. In Paper II, genetic variation of two zinc-

homeostasis genes was studied. Paper III analyzed SNPs in metallothionein genes *MT1A* and *MT2A*.

In the Argentinean Andes, the study area was the village San Antonio de los Cobres and nearby villages, the Salta province, northwestern Argentinean Andes, at an altitude of 3,775 m above sea level. Participants were recruited in 2008 when also blood and urine samples were collected. Cd exposure was probably through food consumption, as there is no industrial pollution and drinking-water Cd-concentrations are low (Concha et al. 2010). At sampling, the participants were interviewed about socioeconomic factors, food consumption, general health, and ethnicity. Based on the interviews, the majority of the study participants were Atacameño Indians, but some also reported a more Hispanic ancestry (Engström 2010).

Blood, plasma and spot urine samples were collected from 203 women. From the first 122 women, additional blood samples were taken for extraction of RNA. Some of the women were first-degree relatives and were, thus, excluded from the genetic association studies. Thus, the studies in Papers I and II comprised 172 women, and among those there were RNA analyzed microarray analysis from 72. A Kruskal-Wallis test revealed that the only significant difference between the total group and the RNA sub-group was median age (36 vs. 34 years).

In Bangladesh, the study area was Matlab, a rural area 53 km southeast of Dhaka. A cross-sectional study was performed in 2002 among women participating in a longitudinal study on health-effects of early-life exposure to environmental pollutants (Kippler 2009; Kippler et al. 2007). All women participating in the study were pregnant. Urine samples were collected in gestational week 8 and erythrocyte and plasma samples in gestational week 14. In Paper I, U-Cd was available for 359 women and Ery-Cd for 235. In Paper II, U-Cd was available for 359 women and Ery-Cd for 400 of the 403 women.

In China, the 512 participants in this study were exposed to Cd at various degrees. The highly exposed group had consumed rice which was contaminated by industrial wastewater until 1995. The concentration in rice had been 3.7 mg Cd/kg. The moderately exposed group consumed rice with 0.5 mg Cd/kg, and the control group consumed rice with 0.072 mg Cd/kg (Jin et al. 1999; Jin et al. 2002). People living in moderately and highly polluted areas were asked to consume commercial rice (0.03 mg Cd/kg). This study was performed in 2006, 11 years after the end of exposure. Volunteers for this study were recruited by the Wenzhou center for disease control and prevention (CDC, Zhenjiang province, China). Background information available to the CDC ensured that the participants were similar with respect to living conditions, social and economic conditions, and lifestyles.

Exposure markers analyzed were B-Cd, and U-Cd (Table 1). Effect markers were UNAG and UB2M (Jin et al. 1999; Liang et al. 2012).

Ethical considerations

The studies included in Papers I and II were approved by the Research Ethics Committees at the Health Ministry of Salta, Argentina, of icddr,b, Bangladesh and Karolinska Institutet, Sweden. Oral and written informed consent was given by all participants before entering the study.

The study in Paper III was approved by the Ethical Committee of Public Health School, Fudan University, in Shanghai, China. Before the sampling, the study subjects gave their informed written consent. The Wenzhou center for disease control and prevention (CDC, Zhenjiang province, China) recruited the volunteers and reported back about the results of the Cd analyses and kidney-function markers.

In none of the three studies, the results of the genotyping were reported back to the participants. Based on published results, and our own studies of the importance of genetic variation for metal uptake and toxicity, we have reason to assume that the genetic effects are of little importance for an individual, but could have impact on group level.

Metal determinations

Cadmium

For the Cd analyses, the erythrocyte samples from the Bangladeshi group and the whole blood samples from the Andean group underwent microwave-assisted acid digestion. The urine samples from both groups were diluted with 1% nitric acid. Urine samples from the Chinese group were acidified with concentrated nitric acid.

The Cd analyses reported in Papers I and II were performed by the same laboratory by inductively-coupled plasma mass spectrometry (ICP-MS; Agilent 7500ce, Agilent Technologies, Tokyo, Japan) with the collision/reaction cell system in helium mode. Limit of detection for B-Cd was 0.011 μg/L for samples from the Argentinean Andes, and for Ery-Cd <0.1 μg/L for samples from Bangladesh. Limit of detection for U-Cd was <0.05 μg/L in both populations. Accuracy was ascertained by reference materials, for which the obtained Cd concentrations showed a good agreement with certified or recommended

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concentrations (Kippler et al. 2007). U-Cd in the Andean and Bangladeshi women was adjusted for specific gravity. In the latter group, U-Cd was available for 359 out of 403 women, both in Paper I and II. Specific gravity adjustment was chosen because the Bangladeshi women were malnourished (Nermell et al. 2008).

Cd analyses in samples from the Chinese men and women were performed by graphite-furnace atomic absorption spectrometry, using standard addition as described earlier (Jin et al. 1999; Jin et al. 2002). Accuracy was ensured by using reference material (Seronorm trace elements urine, Nycomed, Oslo, Norway). U-Cd in Chinese samples was adjusted for creatinine (Cr) concentration.

As Cd was measured in whole blood in the Argentinean Andes and in erythrocytes in Bangladesh, the B-Cd in the Andes was transformed to Ery-Cd, assuming that 95% of the B-Cd is bound to erythrocytes (Nordberg et al. 2007b) and that the density of the erythrocyte preparations was 1.055 g/mL. To account for the volume fractions of erythrocytes and plasma, we used the measured hemoglobin concentrations divided by 340 g/L, which is the mean reference value for hemoglobin in erythrocytes (León-Velarde et al. 2000; Lundh and Öhlin 1991). The median and range of hemoglobin in the Andean women was 156 (90-202) g/L.

Other metals and biomarkers

Analytical methods and their performances (if available) are listed in Table 2.

Table 2. Methods and performance for measurement of zinc and ferritin in plasma (P) and kidney markers in urine (UNAG and UB2M).

Analyte	Population	Method	Limit of detection	Imprecision
P-Zn	Argentinean Andes	Spectrophotometry (accredited method)	0.6 µmol/L	<2.7 %
	Bangladesh	Atomic absorption spectrophotometry	n.a.	<2.0 %)
P-ferritin	Argentinean Andes	Immunoassay (Cobas e601; Roche Diagnostics, Mannheim Germany)	0.5 µg/L	<5.1%
	Bangladesh	Radioimmunoassay (Diagnostic Products, San Diego, CA, USA)	n.a.	n.a.
UNAG	China	Spectrophotometry	n.a.	n.a.[a]
UB2M	China	Enzyme-linked immunoabsorbent assay (ELISA)	n.a.	n.a.[a]

 n.a. not available.

a Samples were analyzed in one batch by the same technician for quality assurance.

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Genotyping

For Papers I and II, so-called tagging SNPs, carrying information about a larger genetic regions, were selected for each of the genes by the use of the software Haploview (Barrett 2009). In addition, rs3811647, rs1799852 and rs2280673 (*TF*) were included based on the reported association with ferritin concentrations (Benyamin et al. 2009), to evaluate whether they would also be associated with Cd concentrations.

Genotyping was performed with Sequenom, a multiplex-PCR (polymerase chain reaction) method based on primer elongation with subsequent detection in a mass-spectrometer. A SNP was accepted when at least 95% of samples were easily distinguished in the mass-spectrometer. Further, SNPs were excluded if they were not in Hardy-Weinberg equilibrium in both populations. A individual was included if there was results for at least 80% of the SNPs. 10% of the samples were repeated and results from both runs were identical.

In Paper III, the SNPs were selected based on the association with Cd concentrations in the kidney (Kayaalti et al. 2010) and health factors that are probably linked with zinc supply (Giacconi et al. 2007; Kita et al. 2006; Mazzatti et al. 2008). The three TaqMan SNP genotyping assays (C__25996927_10 for rs11076161 [*MT1A*], C__60284591_10 for rs28366003 [*MT2A*], and C___1402094_10 for rs10636 [*MT2A*]) were performed with a reaction volume of 5µL. The reaction mix contained for each sample 5 ng DNA, 1 x Universal Master Mix, 0.4 µM of each primer, and 0.1 µM of each probe. 5 % of the samples were repeated and were in agreement with the first run. Genotyping results were evaluated for Hardy-Weinberg equilibrium.

Gene expression

Gene-expression data from a sub-group of 72 of the Andean women (not first-degree relatives) were used in Papers I and II. Peripheral blood was collected in PAX tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland). All samples were frozen and stored at -20 °C, after a maximum of 24 h in room temperature. RNA was extracted with the PAXgene Blood RNA kit (PreAnalytiX GmbH, Hombrechtikon, Switzerland) and stored in -80°C. RNA concentration and purity were evaluated on a Nanodrop spectrophotometer (Wilmington, DE, USA) and RNA integrity (RIN) on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA), showing a good quality (RIN >7.5). For the whole-genome gene-expression analysis, DirectHyb HumanHT-12 v4.0 (Illumina, San Diego, CA, USA) was used, according to the manufacturer's instructions and the analysis was performed at SCIBLU, Lund University. Background signals were filtered from the gene

expression by BioArray Software Environment (BASE; Vallon-Christersson et al. 2009).

Statistical analyses

Each population was treated separately. Hardy-Weinberg-equilibrium was tested by chi-square analysis. Linkage disequilibrium (LD) analysis was performed with Haploview (Barrett 2009) for genes where several SNPs were genotyped.

Associations between genotypes (independent variables) and relative metal concentrations (dependent variables) were examined by multivariable-adjusted linear regression. Each polymorphism was modeled as categorical variable (three genotypes) using the most frequent homozygote in the Bangladeshi women as reference. When the frequency of the minor homozygote genotype was low, this group was pooled with the heterozygotes. Concentrations of the dependent variables were natural log-transformed (ln), in order to achieve normally distributed residuals. All models with U-Cd or Ery-Cd were adjusted for age, as age correlated strongest to Cd concentrations. Adjustments for other potentially influential variables were tested thereafter one by one. In Paper III, associations of genotypes with outcome variables were initially done with analysis of variance (ANOVA) including both - genotype and exposure group as categorical variables. As the Cd concentrations overlapped between the exposure groups, an alternative analysis was performed, where grouping was done in tertiles according to Cd concentrations.

To test for effect modification of the genotypes, a regression model was used with an interaction term between B-Cd or U-Cd and genotype, with UNAG or UB2M as dependent variables (both Cd and kidney variables were natural log-transformed). For those analyses that demonstrated a significant interaction between genotype and ln(B-Cd) or ln(U-Cd), the analysis was stratified for genotype to obtain effect measures for different genotypes.

Further, the influence of *MT* SNPs on the risk of having affected kidney function (measured as having UB2M or UNAG concentrations above 95th percentile of concentrations of individuals from the control area [<80 years]) was analyzed for individuals living in the medium and highly polluted areas. The strength of the associations between genotypes and risk of affected kidney function was estimated as odds ratios with 95% confidence intervals (CIs) by unconditional logistic regression.

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Correlations between metal concentrations and gene expression were made using the Spearman correlation coefficient (r_S). Relations between SNPs and gene expression data were analyzed by the non-parametric Kruskal-Wallis test.

We made many statistical analyses, which urges consideration about potential spurious findings. We primarily focused on those results which were consistent through the material. In addition, in Papers I and II, we used false-discovery-rate (FDR) adjustment separately for each population, and each outcome for the independent SNPs (those that were not in LD [$r^2 < 80\%$]) with the cut-off at the 5% level. This was done with R (version 2.14.2 [http:// www.r-project.org/]). In Paper III there was a different approach, since Wacholder et al. (2004) stated that the false positive report probability is influenced by the significance level, statistical power, and prior probability of the association tested. We concluded that the false positive report probability was low, as the selection of the polymorphisms was based on evidence from other studies, which gave high prior probability. Thus, no formal calculation of FDR was necessary.

RESULTS WITH COMMENTS

Paper I: *TFRC* and other iron homeostasis genes and cadmium concentrations

Carriers of *TFRC* rs3804141 GA and AA genotypes had higher relative U-Cd in both populations than carriers of the GG genotype (Figure 4, and Paper I). The A-allele was found in 26% of the Andean women and in 14% of the Bangladeshi. Asian populations have A-allele frequencies of about 20% (NCBI 2013) indicating
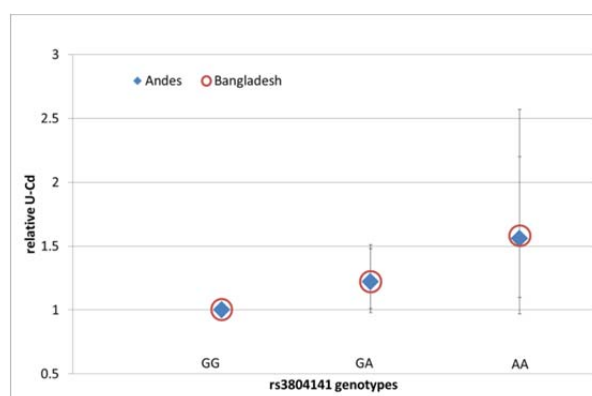

Figure 4. A SNP (rs3804141) in the transferrin receptor gene (*TFRC*) is associated with relative urinary cadmium concentrations.

that effects associated with rs3804141 might also be a concern for several other populations. rs3804141 was included in the study although it deviated from Hardy-Weinberg equilibrium in the Andean group. The SNP was only in weak LD with any other genotyped SNP in *TFRC* ($r^2 < 26\%$). Among the Andean women, the association with U-Cd was more evident in the younger group (<45 years of age) than in the elderly, probably due to an association with regulation of iron metabolism which may differ in pre- compared to post-menopausal women.

In the Andean women only, SNPs of *TF* (rs3811647 and those in LD with it) and of *TFR2* (rs7385804) were associated with U-Cd concentrations (Table 3). The association of rs7385804 with U-Cd remained significant even after FDR adjustment (FDR adjusted p = 0.0096). As the regression analyses were adjusted (besides age) for P-ferritin concentrations, variation in iron status did not explain differences between the Andean and Bangladeshi women. While the genes to be included in the study were selected based on the previously reported link between low iron status and increased Cd biomarkers, it should be noted that none of the SNPs that were found to be associated with Cd concentrations was at the same time associated with P-ferritin. Only one SNP (rs8177186; *TF*) was associated

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with P-ferritin in the Bangladeshi group (75% higher P-ferritin in carriers of the rare TT genotype).

We were not able to detect genotype-specific associations of gene expression with Cd biomarkers, although (1) all of the SNPs affect possible transcription factor binding sites, (2) the gene expression data showed reasonably high levels and range, and (3) there were the expected correlations of gene expression for iron homeostasis.

Table 3. Relative changes of cadmium in urine (U-Cd) depending on *TF* and *TFR2* SNPs in the Andean population. CI=95% confidence interval.

Gene SNP	Genotype	N	U-Cd (CI)	p-value[a]
TF				
rs3811647	GG	32	1	0.009
	AG	82	0.65 (0.50 - 0.86)	
	AA	54	0.76 (0.57 - 1.00)	
TFR2				
rs7385804	AA	114	1	<0.001
	CA/CC	52	0.68 (0.55 - 0.84)	

[a] p-value for linear regression models adjusted for age and P-ferritin.

Paper II: *SLC39A8* and *SLC39A14* and cadmium biomarkers

In the Andean group, carriers of the less common genotypes of four SNPs in *SLC39A8* and *SLC39A14* had higher Ery-Cd compared to carriers of the more common genotypes (Figure 5, and Paper II). In the Bangladeshi group, there was a similar association pattern for three of the SNPs (rs10014145 [*SLC39A8*] and rs4872479, rs870215 [*SLC39A14*]). Those linear regression analyses were only adjusted for age, as the other variables (plasma zinc, plasma ferritin, parity, and BMI) contributed only marginally to the effect estimates.

The minor allele frequencies for rs10014145 and rs233804 (*SLC39A8*) were much higher in the Bangladeshi group (36%), compared to in the Andean group (13% and 8%, respectively). The allele frequencies for rs4872479 and rs870215 (*SLC39A14*) were similar in both populations.
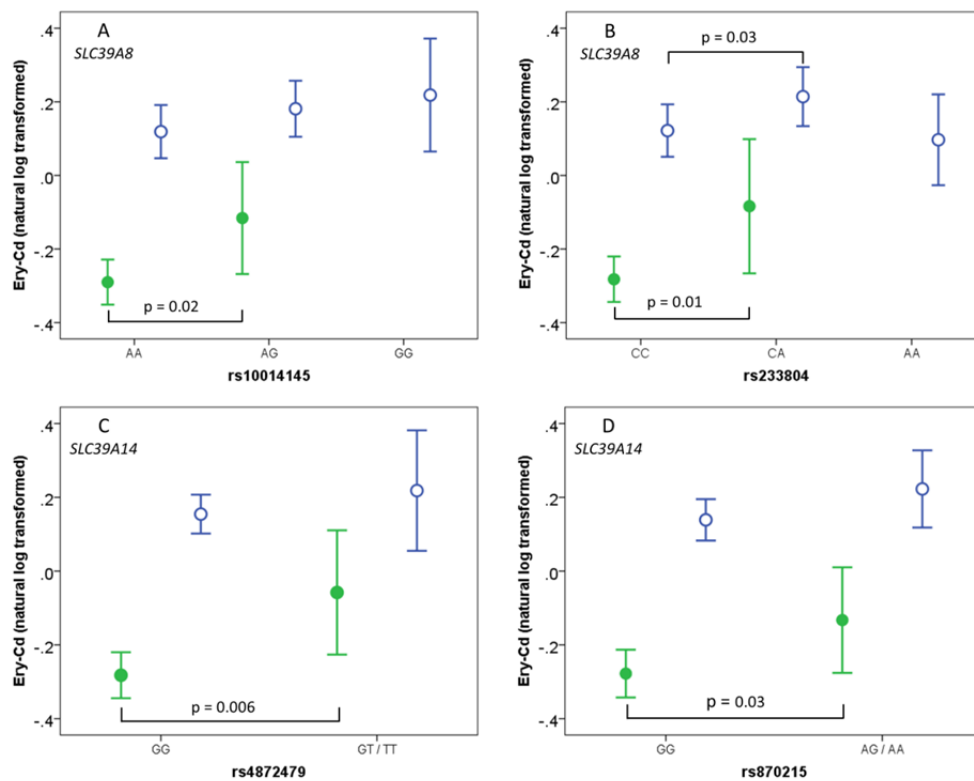
Figure 5. Associations of SNPs in *SLC39A8* (rs10014145 [A]; rs233804 [B]) and *SLC39A14* (rs4872479 [C]; rs870215 [D]) with cadmium in erythrocytes in the Andean (closed circles) and Bangladeshi (open circles) women (symbols show mean values and 95% confidence intervals).

Although non-synonymous SNPs in both genes in general have low minor allele frequencies, we were able to calculate associations with Cd biomarkers for one (rs896378, P33L, *SLC39A14*), but did not find any significant ones. Next, the genotype specific associations of gene expression vs. Cd, plasma zinc and plasma ferritin concentrations were calculated. No associations were detected for *SLC39A8*. For *SLC39A14*, expression was correlated with plasma zinc in the GG genotypes of both, rs4872479 and rs870215. Expression was not correlated with plasma zinc for the heterozygote genotypes (GT and AG, respectively). However, in the latter genotypes, expression of *SLC39A14* was negatively correlated with U-Cd.

Paper III: metallothionein genotype and cadmium-related kidney damage

The main finding of Paper III was the association between *MT1A* rs11076161 and Cd-related kidney toxicity measured as increased concentrations of UNAG and

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UB2M. Carriers of rs11076161 AA and AG genotypes had significantly higher concentrations of B-Cd and UNAG in the high exposure group compared to GG carriers. Genotypes of *MT2A* rs10636 and rs28366003 were only weakly associated with the outcome variables.
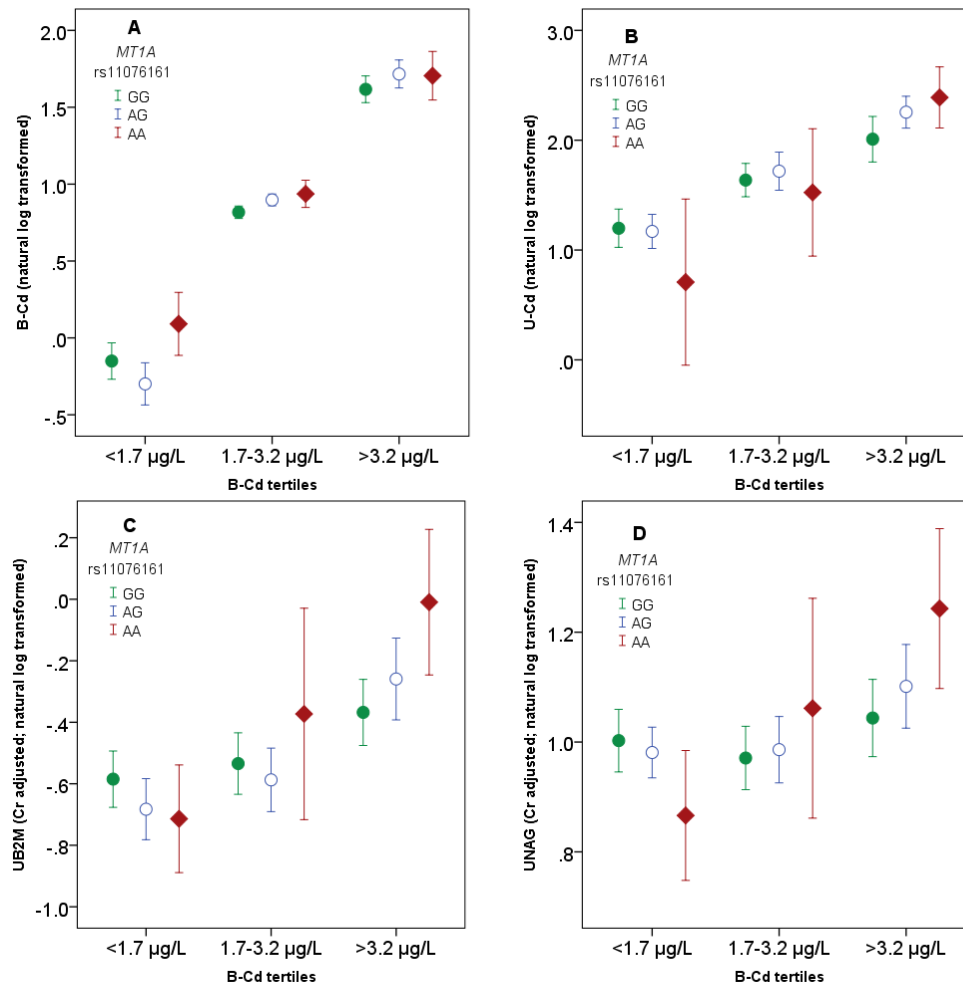
Figure 6. Blood (B-) Cd (A), U-Cd (B), UB2M (C), and UNAG (D) in three exposure categories grouped by tertiles of B-Cd and stratified by genotypes of *MT1A* rs11076161. Symbols show mean values and 95% confidence intervals.

In some of the groups, there were very few carriers of some of the rare genotypes and therefore a problem of low power. As the Cd concentrations in the exposure groups overlapped, stratification was done by B-Cd tertiles instead. This redistributed carriers of the rare alleles more evenly between the groups and thus, somewhat alleviated the power problem. The cut-off values for the lower and

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upper tertiles were of 1.7 and 3.2 µg/L, respectively. This deleted all associations of rs10636 and rs28366003 with any of the four outcome markers and strengthened associations found for rs11076161. Genotype of rs11076161 was significantly associated with B-Cd in the middle tertile (p-value for trend=0.001, both, unadjusted and adjusted for age, sex, smoking) with increasing B-Cd for increasing number of A alleles (Figure 6). Also, rs11076161 was significantly associated with U-Cd in the highest tertile: for increasing U-Cd with increasing number of variant alleles the p-value for trend was 0.01 in the unadjusted model and p=0.001 for the model adjusted for age, sex and smoking. In the alternative analyses by B-Cd tertiles, the genetic association of rs11076161 with UNAG and UB2M was significant in the highest tertile (p-value=0.01 and 0.002, respectively; both p-values for trends in models adjusted for age, sex and smoking).

Genetic effect modification on Cd-related levels of UNAG and UB2M was evaluated by using B-Cd or U-Cd concentrations as continuous exposure markers. First, the interaction p-values were calculated for both, unadjusted and adjusted (sex and age) models. In a second step, the analysis was stratified for genotype to obtain effect measures. The genotype-specific association coefficients of *MT1A* rs11076161 and *MT2A* rs28366003 for their eight exposure-response marker combinations are presented in Table 4.

There was a significant interaction of *MT1A* rs11076161 with B-Cd for concentrations of UNAG (adjusted p<0.001) and UB2M (adjusted p=0.001; Table 4). The association between B-Cd and both kidney function markers had the highest inclination in carriers of the variant genotype AA, lower in AG and the lowest in GG. The interaction of *MT1A* rs11076161 with U-Cd for concentrations of UNAG was not significant and only weak for UB2M (adjusted p=0.062, unadjusted p=0.053). Still, the inclination of the association between U-Cd and UB2M was higher in carriers of the AA genotype compared to GG.

MT2A rs28366003 modified the association between U-Cd and UNAG, where individuals carrying the variant genotypes AA+AG had a lower inclination compared to the common genotype GG. Rs28366003 did not modify the associations between B-Cd and the kidney function markers or U-Cd and UB2M. None of the interactions for rs10636 were significant (data not shown).

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Table 4. Effect modification of the genotypes of rs11076161 (*MT1A*) and rs28366003 (*MT2A*) on the association between on one hand B-Cd or U-Cd and on the other hand UNAG or ln(UB2M) (Cd and kidney variables were natural log-transformed; p-values for interaction adjusted for age and sex; interaction p-values for unadjusted analyses in parentheses). In a second step, the analysis was stratified for genotype to obtain effect measures.

Exposure marker	Biomarkers of renal dysfunction/genotype	Genotype	ß-coefficient	95% CI	p-value interaction
B-Cd	UNAG/	AA	0.55	0.27-0.84	<0.001
	rs11076161 (*MT1A*)	AG	0.15	0.063-0.24	(<0.001)
		GG	0.02	-0.079-0.11	
B-Cd	UB2M/	AA	1.20	0.72-1.6	0.001
	rs11076161 (*MT1A*)	AG	0.45	0.29-0.61	(0.001)
		GG	0.30	0.15-0.45	
U-Cd	UNAG/	AA	0.38	0.22-0.54	0.37
	rs11076161 (*MT1A*)	AG	0.33	0.23-0.42	(0.28)
		GG	0.23	0.14-0.33	
U-Cd	UB2M/	AA	0.57	0.27-0.87	0.062
	rs11076161 (*MT1A*)	AG	0.70	0.52-0.88	(0.053)
		GG	0.39	0.23-0.54	
B-Cd	UNAG/	GG+AG	0.12	-0.01-0-25	0.73
	rs28366003 (*MT2A*)	AA	0.14	0.07-0.22	(0.67)
B-Cd	UB2M/	GG+AG	0.54	0.30-0.78	0.47
	rs28366003 (*MT2A*)	AA	0.44	0.33-0.56	(0.52)
U-Cd	UNAG/	GG+AG	0.16	0.029-0.30	0.018
	rs28366003 (*MT2A*)	AA	0.33	0.26-0.40	(0.013)
U-Cd	UB2M/	GG+AG	0.45	0.20-0.71	0.22
	rs28366003 (*MT2A*)	AA	0.58	0.47-0.70	(0.18)

GENERAL DISCUSSION

Key findings

Paper I found an association between rs3804141, an intronic SNP of *TFRC*, and U-Cd in the two study populations. Twenty six percent of the Andean and 14% of the Bangladeshi population carry an A allele, which is associated with higher U-Cd. Rs3804141 genotypes were not associated with gene expression. There were associations between SNPs of *TF* and *TFR2* and U-Cd, but they were less consistent between the populations.

Paper II found associations of four SNPs of *SLC39A8* and *SLC39A14* with Ery-Cd in the Andean group. The associations were similar in the Bangladeshi group, but not as significant. For the *SLC39A14* SNPs genotype-specific expression might explain the associations with Cd concentrations.

Paper III found an association between *MT1A* rs11076161 AA genotype and B-Cd at high Cd exposure. Also, rs11076161 modified Cd-related excretion of UB2M. Carriers of the AA genotype had higher inclination for the association between UB2M and B-Cd compared to AG and GG carriers. Carriers of rs11076161 variant genotypes (AA and AG combined) had an about three times higher risk to have effects on the kidney function (measured by UB2M), compared to carriers of the GG genotype.

Methodological considerations

Populations

There were several advantages with the two groups of women analyzed in Papers I and II because (1) each of the groups was homogenous with respect to food consumption and socioeconomic status, (2) they represented different levels of Cd exposure with a wide distribution, and (3) there were no other sources of Cd exposure e.g. industrial pollution or smoking. Thus, comparing the two groups permitted to identify outcomes that were associated with the same genetic variation. However, comparing these two groups presented a potential risk, insofar as there are many aspects that also differed greatly between them (some allele frequencies, nutrition, pregnancy, age distribution, etc.), where statistical adjustment could not achieve perfect equality between the groups.

The Chinese group in Paper III was unique as - compared to the other populations - their exposure had been extreme (10-fold), which permitted detection of associations that are not evident at lower exposure levels.

Although the group from the Argentinean Andes was the smallest of the three, the genetic associations were clearer in this group than in the Bangladeshi women. As the group had originally been studied for arsenic exposure (Engström et al. 2011), and the Cd concentrations are relatively low, it was unlikely that there had been any selection with respect to Cd exposure, either in the population or in the sampling.

One aspect that distinguished this group was that they live at high altitude (~ 4,000 m). In order to overcome the condition of constant hypoxia, people in the Andes have higher blood hemoglobin concentrations. There is a genetic basis for this type of altitude adaptation (Beall et al. 1998), which is most prominent in genes controlling erythropoiesis (Zhou et al. 2013). Still, it seems that the higher hemoglobin concentration is mainly achieved by increasing the number of erythrocytes and the hemoglobin content of erythrocytes is the same as at sea level (León-Velarde et al. 2000; Lundh and Öhlin 1991), and therefore it was possible to calculate Ery-Cd from whole B-Cd.

To test the associations between genotypes and Cd concentrations at low iron stores, the groups were split at a P-ferritin of 30 μg/L. In the Andean group, the associations were similar when comparing women with low iron stores to the whole group. Also, at low iron stores, in both, the Andean and the Bangladeshi women, associations between rs3804141 and U-Cd were similar. Thus, the higher iron status of the Argentinean women did not impair the study of genetic associations with Cd.

However, we have to keep in mind that there might have been genetically controlled regulation of zinc and iron needed in erythrocytes that might differ between the Andean group and those living at sea level i.e. the women from Bangladesh, which we were unable to control for.

The Bangladeshi group included in Papers I and II was homogenous, with respect to ethnicity, socioeconomic factors, and absence of industrial pollution which enables differentiating between genetic and other, potentially confounding associations with Cd-exposure markers. The Cd concentrations were higher than in the Argentinean group; still comparisons were possible between the populations because the genetic associations were with relative and not absolute Cd concentrations among the genotypes.

The women in Bangladesh were pregnant and undernourished (as judged by BMI, P-ferritin and P-Zn), which might affect the associations studied. As the main exposure, and, therefore, main concern of toxicological effects was arsenic, it is very unlikely that a selection of the group based on Cd exposure or toxicity had occurred. However, the pregnancy itself could have influenced the metal metabolism, as the risk for exhausting iron stores and thus, increasing Cd uptake, is especially high during pregnancy (Åkesson et al. 2002). Also, the need for zinc is greater, which could either lead to the associations being stronger and, thus to over-interpretation of the results, or that normal associations with zinc status not being evident. Both data for iron (P-ferritin) and zinc (P-Zn) levels were available. Thus, it was possible to – at least to a certain degree – adjust mathematically for iron and zinc concentrations and in turn, effect of the pregnancy. However, these adjustments did in principle not change the main results in Papers I and II.

In China, the volunteers in the highest exposed group had a history of Cd exposure through contaminated rice. The group was compared to a group with lower exposure and a non-exposed one. The cross-sectional study was performed in 2006, ten years after the reduction in exposure. This requires consideration of (1) whether study population was randomly selected, and (2) the time aspect of the biomarkers.

When comparing the allele frequencies for the three SNPs studied with other (Asian) populations in Hapmap (Hapmap Consortium 2007) they are quite similar, indicating that they were representative for the Chinese population.

Persons included had been selected with the support of the local public health authority and, thus, were very similar with respect to living conditions, social and economic conditions and lifestyle. The study was performed about ten years after the maximum Cd exposure in the highly exposed group. For U-Cd this is close to one half-life (Nordberg et al. 2007b). While Cd exposure was reduced, the urinary excretion of low molecular weight proteins, such as B2M or NAG, increased due to aging of the kidney. Therefore, the contribution of Cd toxicity to excretion of low molecular weight proteins might have been underestimated.

Biomarkers

Cd concentrations in blood and urine reported in Papers I and II were analyzed by ICP-MS and all samples were above the detection limit. Accuracy and precision were compated with reference materials and multiple analyses of single samples, respectively. Also, U-Cd analysis results were compared with a different method (GF-AAS; (Kippler 2009). Urinary Cd was normalized to the mean specific gravity of each population in the groups of women included in Papers I and II, while in the mixed population of men and women in China (Paper III), U-Cd was

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normalized to creatinine concentration. Usually, creatinine-adjusted U-Cd represents best the concentration of Cd in the kidney (Åkerström et al. 2013); however, when comparing the malnourished women from Bangladesh to women with a relatively high meat consumption from the Argentinean Andes, density adjustment is to be preferred (Nermell et al. 2008). In the Chinese group, urinary concentrations should preferably have been adjusted for specific gravity, in order to take gender differences in creatinine into account, but those data were unfortunately not available.

Due to the accumulation of Cd in the kidney, U-Cd is mainly a marker of long-term exposure, while B-Cd adjusts to the more recent exposure. As most of the Cd in blood is bound to the erythrocytes (95%; (Nordberg et al. 1985), changes in B-Cd are more rapid than U-Cd, and bound to erythrocyte turnover. Also, there are circumstances, in particular at low Cd levels, when U-Cd does not reflect renal effects, but only variations in proximal tubular re-uptake, also described as reversed causality (Chaumont et al. 2012) which needs to be taken into account when interpreting results involving U-Cd. In Paper III, effect modification of the genotypes of rs11076161 (*MT1A*) on the association between B-Cd and kidney effects was clearer than effect modification on the association between U-Cd and kidney effects. Therefore reversed causality was of minor importance for the interpretation of results.

Iron status is generally measured by P-ferritin. These are standard analyses and have been done with high precision in the two groups of women (Papers I-II). The analyses were performed by two different methods (Argentinean Andes immunoassay; Bangladesh radioimmunoassay). However, the groups were treated separately in the statistical calculations, and, therefore, this did not cause a problem for the interpretation.

Zinc in plasma was measured by spectrophotometry in the Andean women and by atomic absorption spectrophotometry in the Bangladeshi. Both methods were very precise (imprecision <2.7% [Andes] and <2.0% [Bangladesh]). All samples were well above the limits of detection. There has been some discussion whether zinc in plasma or in blood would be the better marker (Lowe et al. 2009; Sandstead and Au 2007). Compared to plasma ferritin as a mobile store of iron, stored pools of zinc are very small and, thus, zinc turnover is faster (King 2011). In addition, there is strict homeostasis. Increased zinc in plasma from food intake would be rapidly transferred to the tissues in need, and, thus, the concentration in plasma would return to the "normal" level. Also, immune response and wound repair are processes that require extra zinc and, thus, deplete zinc in plasma (Liuzzi et al. 2005). Due to these rapid regulatory processes, associations with genotypes and zinc concentrations are hampered.

Genotyping and gene expression

The selection of SNPs was based on information available from the International Hapmap project. It has in its initial phase collected data about genetic variation from four populations with African, Asian, and European ancestry (Hapmap Consortium 2003). As allele frequencies of genetic variations differ between populations and we assumed that the allele frequencies in the study populations are closer to persons with Asian than persons with European ancestry, the selection of TagSNPs in Papers I and II was based on Hapmap data for Asian population groups. Two main hypotheses for functionality of SNPs were followed: (1) a SNP could either change the protein (non-synonymous SNP) and thus, the affinity of the protein to Cd or (2) a SNP could modify gene expression. In order to investigate whether non-synonymous SNPs were associated with Cd metabolism, a number of these were included in the study. However, it turned out that most of the non-synonymous SNPs are very rare in the population groups in Papers I and II and therefore no statistical analyses were possible, which was a limitation of these studies. Also, some of the predicted TagSNPs turned out to have no variants or too low minor allele frequency for statistical analyses. Some of these analyses were still possible by pooling the rare homozygote genotype with the heterozygotes. For genes with results for many SNPs, LD-plots were calculated with Haploview. While some SNPs were in LD, the selection of SNPs still seemed to be adequate.

In populations that are in equilibrium, the Hardy-Weinberg law can be used to check for the quality of the sampling/genotyping. Thus, if a SNP is not in Hardy-Weinberg equilibrium we conclude that non-random sampling has occurred or one of the alleles has been preferably amplified in the genotyping process. SNPs that were not in Hardy-Weinberg equilibrium in both, the Andean and the Bangladeshi population were excluded. As the quality of the genotyping was good (duplicate genotyping for 5% of the samples with identical results and recognition of 90% of the samples per SNP) we have to acknowledge that, by chance, non-random sampling might have occurred for some of the SNPs. Those SNPs that were in Hardy-Weinberg equilibrium in one of the populations but not in the other were nevertheless included.

It might be possible that an unmeasured variant in LD with the SNP is responsible for the observed association with U-Cd. The original selection of SNPs was based on tagging-SNPs in Asian population. After the genotyping, the actual LD was calculated for the study groups in some of the genes. Associations with Cd biomarkers were very similar for SNPs that were in LD. Therefore it seems

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unlikely that associations for an unmeasured variant would deviate much. It could, however, change the mechanistic interpretation of the results.

For a sub-group of Andean women, there were data on whole-genome gene-expression. RNA was extracted from whole blood as the only tissue available for RNA analysis. Moreover, we considered this tissue relevant, given the fact that biomarkers in blood and plasma were analyzed in relation to genetics. Later, the gene expression database BioGPS (Wu et al. 2009) and The Human Protein Atlas (Uhlén et al. 2010) were used to confirm expression and protein levels. As it turned out, expression levels for most of the genes of interest are very low in whole blood and the expression levels in whole blood are not very representative of the expression in the main target tissues for each gene. Also, when looking at genotype specific gene expression data, the groups were very small which additionally impeded the interpretation. However, despite the low level of expression, the data were useful and some associations between genotype specific expression and Cd concentrations were found.

Statistics

As to Papers I and II, we considered the possibility to use one of the populations to generate hypotheses and the other to test these. However, the populations were too different in background factors to allow such an approach. Therefore, we genotyped the same SNPs in both populations and compared the populations to check for common patterns.

We took advantage of previously collected samples. Thus, population size was predetermined and not included in the planning of this thesis. Hence, the studies encompassed some problems with statistical power. For example, the Andean population (Papers I and II) was fairly small, especially for specific genotypes and when gene expression was studied. As regards the Bangladeshi (Papers I and II) and Chinese (Paper III) populations, the situation was more favorable.

We made many statistical analyses, which urges consideration about potential spurious findings because of multiple inference. According to Wacholder et al. (2004), prior evidence is one of the strongest determinants whether findings are true of false. In Paper III, the *MT*-SNPs were chosen based on prior evidence from literature. However, in Paper I the reviewers requested a mathematical correction, which was done by the "False-Discovery-Rate" (FDR) procedure and later also applied in Paper II. As mathematical corrections refer to the number of independent tests and it turned out that in our two groups of women, some of the polymorphisms were partly in LD, and the tests therefore not independent, we reduced the number of tests by excluding SNPs that were in LD ($r^2 > 80\%$) and tested the populations independently and the major results became non-significant

after FDR adjustment. We still considered the findings true if they were consistent between the populations.

Confounding by any variable associated with both the genotype and the outcome (Cd concentrations) is not very likely to occur within the populations, but this could very well be the case when analyzing populations combined and therefore it was very important to keep the groups apart for the statistical analyses. Effect modification by other influential variables, like iron and zinc status, age, gender, and smoking have been adjusted for in the multivariate models. There is a theoretical possibility that other genes, which we did not study, were associated with the ones we studied and with Cd metabolism.

The study design of all three studies was cross-sectional. In epidemiology, this is usually seen as a problem when trying to reach conclusions regarding causality. However, it is difficult to identify factors, which would cause differential selection or confounding (see above) of the relationship between genotype and biomarkers of Cd retention, i.e. the major problems in cross-sectional studies. Thus, it should be possible - with reasonable certainty - to assume that the associations between genotypes and Cd metabolism were causal.

Overview of the research area

Below, a selection of studies of gene-environment interaction for Cd and - if available - functionality of SNPs is listed (Table 5). Those, and others, will be used in the next section to help in interpreting the findings in this thesis.

Table 5. Published studies about gene-environment interaction for cadmium (Cd) and functionality of SNPs (if available).

Gene / SNP	Reference	Study type	Outcome variable	Result
TFRC - transferrin receptor (p90, CD71)				
	Honda et al. (2013)	Animal study (mice)	Gene expression in brain after Cd exposure	*TFRC* showed higher expression after Cd exposure.
	Cucu et al. (2011)	Cell culture (primary human tubular cell)	Gene expression after Cd exposure	*TFRC* showed higher expression after Cd exposure.
TFR2 - transferrin receptor 2				
rs7385804	Pichler et al. (2011)	GWAS[a] Cross-sectional and Meta-analysis	Associations with serum iron	rs7385804 was associated with serum iron.
rs7385804	Ganesh et al. (2009)	GWAS Cross-sectional and Meta-analysis	Associations with erythrocyte traits	rs7385804 was associated with hematocrit.
rs7385804	Soranzo et al. (2009)	GWAS Cross-sectional and Meta-analysis	Associations with hematological parameters.	rs7385804 was associated with red blood-cell count.
TF - transferrin				
	Dietrich et al. (2011)	Cell culture (Carp spermatozoa)	Motility after Cd exposure with or without addition of transferrin	Cd bound to transferrin; addition of transferrin protected motility from toxic effect of Cd exposure.
	Saljooghi and Fatemi (2010)	In vitro study	Cd binds to transferrin	In plasma, about 50% of the Cd was bound to transferrin.
	De Smet et al. (2001)	Animal experiment	Cd binds to transferrin	In plasma, a high portion of Cd was bound to transferrin (much higher than in humans).
	Harris and Madsen (1988)	In vitro study	Cd binding to human transferrin	Cd binds to human transferrin.
rs3811647	Benyamin et al. (2009)	GWAS Cross-sectional	Associations with transferrin and ferritin	rs3811647 was associated with ferritin.

Table 5. Continued

rs3811647	Constantine et al. (2009)	GWAS Cross-sectional	Associations with transferrin and ferritin	rs3811647 was associated with ferritin.
rs3811647	Pichler et al. (2011)	GWAS Cross-sectional and meta-analysis	Associations with ferritin	rs3811647 was associated with ferritin.

SLC11A2 - solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (DMT1)

	Öhrvik et al. (2013)	Cell culture (human Caco-2)	Gene expression in relation to Cd exposure	*MRP1* upregulated but not *DMT1 (SLC11A2)* or *FPN (SLC40A1)*
	Illing et al. (2012)	Cell culture (Xenopus oocytes)	Transport of metals	*DMT1 (SLC11A2)* transported Cd.
	Gu et al. (2009)	Animal study (rats)	mRNA and protein levels of DMT1 in parts of the brain upon Cd and/or lead (Pb) exposure	mRNA unchanged; higher protein levels of DMT1 in the Cd + Pb group and higher metal concentrations.
	Bressler et al. (2004)	Review		Summarises role for DMT1 (*SLC11A2*) in Cd transport.

SLC40A1 - solute carrier family 40 (iron-regulated transporter), member 1 (ferroportin 1)

	Troadec et al. (2010)	Cell culture (several cell types)	FPN1 (*SLC40A1*) mRNA levels upon Cd exposure.	The mRNA levels increased.
	Park and Chung (2009)	Cell culture (mouse macrophages)	FPN1 (*SLC40A1*) mRNA levels upon Cd exposure.	The mRNA levels increased.

SLC39A8 - solute carrier family 39 (zinc transporter), member 8, (alternative ZIP8)

	Schneider et al. (2013)	Animal study (transgenic mice)	Cd in kidney	Cd concentration in kidney increased with higher expression of *SLC39A8*.
	Fujishiro et al. (2013)	Cell culture (RBL-2H3)	Cd concentration, LD50 values	Cd concentration decreased with lower expression of *SLC39A8*, LD50 increased.
	Napolitano et al. (2012)	Cell culture (human lung epithelia and A549 cells)	Cd concentration, cell death	Higher Cd concentration with higher *SLC39A8* expression and higher cell death rates.

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Table 5. Continued

	Reference	Study type	Outcome measure	Finding
SLC39A8	Fujishiro et al. (2009a)	Cell culture (Cd-resistant mouse cells)	Cd accumulation	Suppressing *SLC39A8* expression reduced Cd uptake to 35%.
	Fujishiro et al. (2009b)	Cell culture (A7 cells)	SLC39A8 methylation and expression level	Hypermethylation of the CpG island of *SLC39A8* resulted in lower expression level and lower Cd toxicity
	He et al. (2009)	Transgenic mice	SLC39A8 expression and renal function	Renal failure because of Cd toxicity in mice with high expression of *SLC39A8* in proximal tubular cells.
	Himeno et al. (2009)	Review		Summarises the association between *SLC39A8* expression and Cd uptake
	Aiba et al. (2008)	Cell culture (Small-cell lung cancer cells)	Cd accumulation	Downregulation of *SLC39A8* expression reduced Cd uptake.
	Liu et al. (2008)	Cell culture (Xenopus oocytes)	Transport characteristics	Cd transport by SLC39A8 is faster than zinc transport. Cd and zinc compete for the transporter.
SLC39A14 - solute carrier family 39 (zinc transporter), member 14, (alternative ZIP14)				
	Min et al. (2013)	Animal study (mice)	Liver Cd concentrations and SLC39A14 expression	Higher liver Cd concentrations with higher *SLC39A14* mRNA levels.
	Fujishiro et al. (2012)	Cell culture	Kidney proximal tubular uptake by SLC39A8 and SLC39A14	Expression of both transporters contributes to Cd re-uptake in the kidney.
	Jenkitkasemwong et al. (2012)	Review		Summary of tissue distribution of *SLC39A8* and *SLC39A14*
	Girijashanker et al. (2008)	Cell culture	Transport characteristics	Cd and Zn compete for transport by *SLC39A14*.
MT1A/ MT2A - metallothionein1A and metallothionein2A				
	Nordberg et al. (2007b)	Overview book chapter		
	Boonprasert et al. (2012)	Cross-sectional	MT expression	
	Huang et al. (2013)	Cross-sectional	MT expression and kidney function	*MT1A* and *MT2A* mRNA expression increased in blood in line with Cd concentrations

Table 5. Continued

MT1A rs11076161	Zavras et al. (2011)	Case-control	Oral squamous cell carinoma	A allele protective
MT1A rs11076161	Yang et al. (2008)	Case-control	Type 2 diabetes with neuropathy	Significant association
MT2A rs28366003	Forma et al. (2012)	Case-control	Prostate cancer	Higher risk for G allele
MT2A rs28366003	Krześlak et al. (2013)	Case-control	Prostate cancer Gene expression Cd concentration	G allele = risk G allele = lower expression Different between cases/controls, but no association with expression
MT2A rs28366003	Kita et al. (2006)	Cell culture (HEK293)	Transcription	Less efficient transcription of G allele upon Cd exposure
MT2A rs28366003	Kayaalti et al. (2010)	Cross-sectional	Cd in renal cortex	Higher Cd concentrations associated with G allele
MT2A rs28366003	Kayaalti et al. (2011)	Cross-sectional	Cd in blood	Higher Cd concentrations associated with G allele

[a] GWAS = Genome-wide association study.

Mechanistic interpretation

A summary of earlier epidemiologic and experimental work is given in Table 5 and some of the findings are commented upon below in relation the findings of the thesis.

There is strong evidence from epidemiologic studies of a link between low iron stores and increased Cd concentrations (Bárány et al. 2005; Gallagher et al. 2011; Kippler et al. 2007; Meltzer et al. 2010; Skerfving et al. 1999). Several of the iron-related proteins have been shown to bind Cd (DMT1 / *SLC11A2*, Bressler et al. 2004, transferrin / *TF* Harris and Madsen 1988), or the genes to be differentially expressed upon Cd exposure *(SLC40A1* Troadec et al. 2010*; TFRC* Cucu et al. 2011) which justifies their inclusion in Paper I.

The main finding of Paper I was an association between *TFRC* rs3804141 with U-Cd in both study populations. Higher U-Cd was associated with increasing numbers of A alleles. Besides the extremely high levels in bone marrow, *TFRC* is also present in nearly all other tissues, among them the kidney, at about 20% of the expression level in bone marrow (Uhlén et al. 2010). For a *TFRC* SNP to be associated with U-Cd, but not with B-Cd, as was the case in Paper I, the interaction of the phenotype with Cd exposure would have to be in the kidney (Figure 2).

We did not find any association between *TFRC* rs3804141 genotypes and gene expression in whole blood. It is still possible that the association with Cd markers is based on genotype-specific expression in other tissues, e.g. the kidney. The studies by Cucu et al. (2011) and Honda et al. (2013) confirm that *TFRC* expression is increased upon Cd exposure. Indeed, it would only be possible to elucidate the mechanism for rs3804141 in cell cultures of erythrocyte precursors or kidney proximal tubular cells.

In the iron metabolism, *TFRC* acts in concert with *TFR2* to regulate intracellular iron by delivering it from transferrin into the cytoplasm (Wang and Pantopoulos 2011), as depicted in erythrocyte precursor cells in Figure 2. There was a population specific association between *TFR2* rs7385804 in the Andean group, where the C-allele was associated with lower U-Cd. Although only evident in the Andean group, the association was sufficiently strong to survive adjustment for multiple comparisons. Functionality of the SNP has been demonstrated by its association with lower serum iron (Pichler et al. 2011), lower hematocrit (Ganesh et al. 2009), and with red cell count (Soranzo et al. 2009). The association of rs7385804 with markers of iron metabolism suggests a true association of

rs7385804 with U-Cd. However, this should be confirmed, because the SNP was not in Hardy-Weinberg equilibrium in the Andean group.

Another population-specific association was between variant genotypes of several *TF* SNPs in linkage disequilibrium with rs3811647 and lower U-Cd in the Andean group. That Cd can and does bind to transferrin has been shown both *in vitro* (Harris and Madsen 1988; Saljooghi and Fatemi 2010), in cell cultures (Dietrich et al. 2011) and animal experiments (De Smet et al. 2001). Although the binding of Cd to transferrin is stronger in animals, there is still a significant portion bound in humans. With respect to iron metabolism, the functionality of rs3811647 has been shown (Benyamin et al. 2009; Constantine et al. 2009; Pichler et al. 2011). Taken together, an association of the *TF* SNP with Cd seems likely.

Among the iron metabolism genes/proteins *SLC11A2*/DMT1 has a strong role in intestinal Cd uptake, based on the findings by Illing et al. (2012), Gu et al. (2009) and many previous studies, reviewed by Bressler et al. (2004); Figure 2). Also, the expression of *SLC40A1* (FPN1) in relation of Cd exposure in cell cultures seems clear (Park and Chung 2009; Troadec et al. 2010). In spite of these facts, no association between any of the 17 SNPs genotyped in these genes in the Bangladeshi and Andean women and Cd concentrations were detected. As both genes/proteins act in the intestinal Cd absorption, associations of SNPs with B-Cd should have been detectable. However, a recent experimental study with cultured human intestinal cells indicated a weaker role of both transporters for Cd uptake (Öhrvik et al. 2013); however, the cells were immature.

The fact that the observed associations of *SLC39A8* and *SLC38A14* SNPs were stronger for Ery-Cd than for U-Cd could have several reasons. Association with intestinal absorption rate of Cd is possible for *SLC39A14* SNPs (Figure 3) which is expressed to cover situations of additional need for zinc e.g. in response to inflammation (Cousins 2010; Liuzzi et al. 2005). Also association with Cd uptake into erythrocytes would be possible for SNPs of both genes, as they are present in bone marrow (Uhlén et al. 2010) and, consequently, in erythrocyte membranes.

Studies including *SLC39A8* and *SLC39A14* and their association with Cd, as listed in Table 5, have mainly been performed in cell cultures and animals and have looked at variation in gene expression as outcome.

We found some evidence that genotype-specific expression is the underlying mechanism for the association of *SLC39A14* SNPs and Cd biomarkers. The expression levels of *SLC39A14* in blood and kidney are relatively low (Jenkitkasemwong et al. 2012; Nomura et al. 1994). Still, we might speculate whether genotype specific expression in the kidney was responsible for the

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observed inverse association with U-Cd. As *SLC39A14* is an ingoing transporter, the expression in proximal tubuli and, consequently, the involvement in re-uptake of Cd would lead to an inverse correlation with U-Cd.

Paper II did not find any association of genotype-specific expression of *SLC39A8* in whole blood and Cd biomarkers. This seems justified as the corresponding protein (ZIP8) in erythrocytes of mice was found to be non-responsive to zinc status (Ryu et al. 2008). It seems that *SLC39A8* expression is regulated in developing cells but is at a fixed level in mature ones as found in whole blood.

Of all proteins/genes associated with Cd metabolism, metallothioneins are probably the most well-studied ones (Boonprasert et al. 2012; Huang et al. 2013; Nordberg et al. 2007b). The focus for the *MT1A* SNP interaction with Cd toxicity is on the kidney (Figure 3). The *MT* SNPs studied in Paper III did affect putative transcription-factor binding-sites and, thus, hinted at genotype-specific transcription being the mode of action. No associations between rs11076161 and Cd or kidney function were reported previously. We interpreted associations of the SNP with other health factors as supporting evidence for the functionality of the SNP and, thus, supporting our findings. Hence, (Zavras et al. 2011) found an association with oral squamous-cell carcinoma and (Yang et al. 2008) with type 2 diabetes with neuropathy. In relation to cancer, genotype-specific expression, maybe via an epigenetic mechanism, of *MT1A* through its interaction with cancer-suppressor genes may be the underlying mechanism for functionality of rs11076161 genotypes. The association with diabetes complications may be through genotype-specific expression in oxidative stress pathways. However, currently the mechanistic interpretation of the function of the *MT1A* SNP is based on circumstantial evidence.

One other SNP was studied in Paper III, but only weak associations with B-Cd were detected. Kayaalti et al. (2010) found that carriers of the heterozygote and rare homozygote genotypes of a SNP (-5 A/G; rs28366003) in the promoter of *MT2A* had higher mean Cd concentration in kidney cortex and higher B-Cd (Kayaalti et al. 2011). Concerning association of *MT* SNPs and toxikokinetics of Cd, two studies found that rs28366006 genotypes were associated with prostate cancer (Forma et al. 2012; Krześlak et al. 2013) and the risk allele (G) was, at the same time, associated with lower expression of *MT2A*. While Cd levels differed significantly between tissue from cancer cases and controls, there was no genotype-specific association. Kita et al. (2006) had previously found reduced expression of this G variant in response to Cd and Zn exposure. In Paper III, only weak associations between the genotypes and B-Cd were detected. Also, carriers of the G allele had lower UNAG. The apparent contradiction between those findings could be explained by cell type specific differences.

Practical significance and relevance

There is little information on which genetic factors that affect the metabolism and toxic effects of Cd, i.e. gene-environment interactions. The present work has supplied information on what genes, and more specifically polymorphisms, affect the accumulation of Cd in the body, and thus, their potential to cause toxic effects.

Knowledge about gene-environment interaction is important, because it can explain differences in sensitivity between individuals and populations, together with other effect-modifying factors, e.g. sex, age, smoking, and nutrition.

Gene-environment interactions as regards toxicokinetics and toxicodynamics may have relevance on different levels. Thus, they may call for attention by the health-care system, in order to identify subjects with particular vulnerability, and take actions, e.g. by advice in order to reduce the exposure through changes of lifestyle.

For Cd, even a slight impact of genes on toxicokinetics may be of importance, because turnover is very slow (biological half-time decades). However, even so, the present effects of Cd, as identified in Papers I, II and III, are not sufficiently strong to call for interventions on an individual level.

However, there is a second aspect - that gene-environment interaction may require attention in the risk-assessment procedure. Then, gene-environment interaction can impact two steps: First, in those cases where biomarkers are used as an index of exposure and risk, the genetic influence on toxicokinetics may require consideration, since it will affect the relationship between exposure and concentrations in the biomarker indices (blood, urine, etc.).

Second, when toxicodynamics is modified, the shape of the dose-response curve can be affected, i.e. the relationships between exposure and effects, and between biomarkers and effects. There may be a particularly sensitive fraction in a population, which should be considered. Also, differences in gene frequencies between populations might mean that they should require separate risk assessments. Further, caution should be taken when merging information from different populations in risk-assessment schemes. Such differences in gene frequencies have been shown for, e.g. lead (Pawlas et al. 2012; Zheng et al. 2011), and arsenic (Schlebusch et al. 2013), but have still not been taken into account by risk-assessment bodies, e.g. European Union (European Food Safety Authority; EFSA) and Food and Agricultural Organization (FAO) or World Health

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Organization (WHO) (Joint FAO/WHO Expert Committee on Food Additives, JECFA). Both have recently published risk assessments for Cd, in which information from different populations have been pooled in a benchmark approach (EFSA 2009, 2011; JECFA 2010).

CONCLUSIONS

1. **Variation in iron genes is associated with cadmium metabolism.**
2. **Specifically, variation in the *TFRC* gene is associated with cadmium metabolism. The consistency between two widely different populations hints at a causal association.**
3. **The *TFRC* variation does not influence cadmium metabolism by modifying gene expression in blood.**
4. **Variations in other iron genes are less clearly associated with cadmium metabolism.**
5. **Variation in zinc genes is also associated with cadmium metabolism.**
6. **Possibly modification of expression of the zinc-associated gene *SLC39A14* gene may be the mechanism for the association with cadmium metabolism.**
7. **Variation in metallothionein genes is associated with cadmium metabolism and toxicity at high exposure, less likely at low.**
8. **The mechanism behind modification of cadmium toxicity is possibly via an effect on cadmium metabolism.**

FUTURE RESEARCH QUESTIONS

In-vitro studies
- A *TFRC* functional study in an *in-vitro* system would be helpful to verify the present findings, and to close the gap to conclusions about causality. For example, exposure to Cd of human bone-marrow mononuclear cells (erythrocyte precursors), either from donors with different genotypes, or transfected cells, would elucidate the role of this transport gene and whether modification of gene expression is the mode of action for the intronic polymorphism.

In-vivo studies
- Experiments with animals could help to elucidate the mechanisms behind the associations for the present SNPs with toxicokinetics and toxicodynamics of Cd. Animal experiments would permit to examine tissue-specific gene-expression patterns. Then, species-differences in Cd toxicity must be taken into account.
- In a hypothesis-driven approach, tissue-specific gene-expression patterns could reveal interactions of low molecular weight proteins with the cubilin/megalin complexes for variations in Cd re-uptake in the kidney proximal tubuli.

Epidemiologic studies
- The existing genome-wide association study data should be explored more in-depth.
- The SNPs with strongest associations with Cd should be genotyped in other populations, preferably with high frequency of the minor allele.
- *SLC39A8* and *14* should be better characterized, both for tagging and for function of SNPs in relation to Cd (and zinc).
- Toxicodynamics should be better explored in populations with varying exposure to Cd and zinc, age and genetics. Then effect parameters, e.g. kidney function or bone-mineral density, should be available.

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