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PHIME

Public health impact of long-term, low-level mixed element exposure in susceptible population strata

Instrument: Integrated Project
Thematic Priority: Priority 5, Food Quality and Safety

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Contents

Summary

PHIME (Public health impact of long-term, low-level mixed element exposure in susceptible population strata) was an Integrated Project within EU´s Sixth Framework Programme for Research & Technological Development. It ran 2006-2011 with 35 Partners in 22 countries.

The background was a renewed interest in toxic metals, due to a growing awareness that the exposure in the general population in Europe and elsewhere is at levels with potential to cause toxic effects in susceptible individuals. Such exposures may have a role in the etiology of common clinical diseases, as well as sub-clinical effects, which may be serious for the society. Further, novel methods (analytical, effect markers, molecular biology and epidemiology) had been developed, which facilitated better assessment of health risks.

The overall aim was to improve assessment of the public-health impact of toxic and essential metals via food, addressing the complexity of exposures, interactions (e.g. with nutrition), and risk groups (women, children, the elderly and individuals with genetic susceptibility). PHIME also addressed mechanisms for uptake of metals in plants, and thus into the human food chain.

PHIME focused on producing scientific data, through close cooperation between research groups with knowledge, methods and study groups that complement each other. A closely cooperating working party was formed, which planned and executed collaborative studies and discussed findings, resulting in, up to date, 181 articles in peer-reviewed (most international) journals; seven are in press. Much additonal data is collected and will be published soon.

A wealth of important, novel information has been produced. For example, the exposure to lead and cadmium seems to be fairly similar in many Europan countries, with the exception of particularly polluted areas. The exposure to lead and mercury is decreasing, while cadmium does not show such a favourable time trend. The tissue concentrations of "catalytic converter elements" platinum, palladium and rhodium are much lower than previously thought.

The toxic effect of methylmercury on the central nervous system of fetuses and the myocardium of adults is markedly modified by nutrition. Arsenic and manganese, ingested mainly through drinking water and food, affect development and health of fetuses, infants and children. Lead exposure is toxic to children´s central nervous system at very low exposures.

Data cast doubt on the excretion of low-molecular weight proteins as a biomarker of cadmium risk at low exposure, but, at the same time, there is evidence of low-level cadmium exposure causing toxic bone effects, with decrease of bone mineral density, increase of osteoporosis and fractures. Preventive actions are needed, in light of the continuous exposure world-wide.

PHIME has also profoundly increased the understanding of molecular mechanisms for the uptake of metals in plants, which will enable breeding of cereals with increased levels of essential elements, and - hopefully - decreased toxic.

Gene-environment interaction is important in metal toxicity. Thus, the metabolism (toxicokinetics) of mercury, arsenic, and lead is modified by genetics, as is toxicodynamics of arsenic, lead, cadmium and manganese. This should be considered in risk assessment, as the risk may vary between individuals, and between populations with different gene frequencies.

Taken together, the scientific results contributed by PHIME have brought new, important insights to the health impact from metals, as a basis for risk assessment and prevention.

Goals achieved

PHIME - the Project

PHIME (Public health impact of long-term, low-level mixed element exposure in susceptible population strata) was an Integrated Project (IP) within the European Union´s Sixth Framework Programme (FP6) for Research & Technological Development (Thematic Priority Food Quality and Safety). PHIME ran 2006-2011, with an EU budget of 13.4 MEUR. Already during year 1, PHIMETTC (Targeted Third Countries) was amended to PHIME. The combined project involved 35 partners in 22 countries (of which five non-European).

The background of PHIME was a renewed interest in toxic metals, due to a growing awareness that the exposure in the general population in Europe and elsewhere was close to - or even above - levels with potential to cause toxic effects in susceptible individuals. As a consequence, metals may play a role in both the etiology of common clinical diseases and for sub-clinical effects. The cumulative impact of the latter effects may be very serious for the society, though not of major importance for the individual. Further, novel methods had become available (analytical, effect markers, molecular biology and epidemiology) for better assessment and prevention of health risks.

Accordingly, the overall aim of PHIME was to improve integrated public-health risk-assessment of exposure to toxic and essential metals via food, addressing the complexity of exposures, interactions, risk groups (including women, children, the elderly and individuals with genetic susceptibility), nutrition, and mechanisms of action. PHIME also addressed mechanisms for uptake of metals in plants, and thus into the human food chains. This enables preventive measures to be taken in order to reduce the human burdens of toxic metals and their consequences for health and development.

PHIME has been focused on the production of new scientific data, to a large extent by favouring close cooperation between research groups with knowledge, methods and study groups that complement each other. A large number of the research groups in Europe involved in the area were engaged in the work, and in addition several of the most prominent research groups outside Europe. PHIME has also collaborated with sister EU projects. During the project period, a closely cooperating working party was formed, with intensive workshops in sub-constellations at least twice a year to plan and execute collaborative studies, and discuss the results. There were also many activities inbetween. The network is now planning for future cooperative activities.

The close cooperation between Partners within WPs often meant a need of transfer of biological samples over national borders, from sampling sites to analysing laboratories. One aspect, which was not clearly realized initially, was that laws and regulations sometimes obstructed, or even prohibited such cooperation. Thus, in future projects, such aspects should be addressed early during planning.

During 2006-2011, 181 articles have been published in peer-reviewed (mostly international) scientific journals; seven are in press, 31 submitted and 14 in preparation (manuscripts). A wealth of additonal data has been collected and is under processing, to be published soon.

In the following, the presentation of overall achievements will follow the original goals as presented under their three global headings, with their sub-objectives. In the later part of this report, the presentation will follow the individual WPs.

Objectives of PHIME

There were three overall questions of PHIME, as tackled under its four Pillars:
1. What are the problems (Pillars I and II)?
2. Where are the problems (Pillar III)?
3. Possible solutions of the problems (some; Pillar IV, and some horizontal WPs).

Several aspects have also been addressed in horizontal WPs, each covering two or more Pillars.

The specific original objectives under these three main headings were:

What are the problems (Pillars I and II)?

i) We will epidemiologically assess the impact of toxic metal (particularly cadmium, mercury, lead and manganese) exposure through foods on diseases of public health concern (nervous and cardiovascular systems, osteoporosis/fractures, kidneys, diabetes). Some studies will utilize unique biobank material. Occupational exposures will be studied as models.

ii) Particular interest will be paid to interaction between toxic elements (cadmium, mercury, lead and manganese) in mixed exposures (several metals and other pollutants, like DDT, PCBs and Ochratoxin A).

iii) We will characterize benefits of exposures to essential elements (selenium, zinc)/other dietary components (fatty acids, fibre), and describe some aspects of risk/benefit relationships.

iv) We will address pathomechanisms and gene-environment interactions.

v) We will focus on susceptible groups (fetuses/infants/children, fertile women and elderly, nutrition and gene-environment interactions), as regards metabolism and toxic effects.

B. Where are the problems (Pillar III)?

vi) We will develop and validate new methods for biomonitoring of exposures.

vii) We will define geographical patterns/sources of exposure to cadmium, mercury, lead, platinum, palladium and rhodium in EU Member States and some other areas (Ecuador, Morocco, China), especially in children and women.

viii) We will assess time trends of exposure, retrospectively and prospectively.

C. Possible solutions to the problems (Pillar IV and some cross-sectional WPs)

ix) We will develop probabilistic models, describing the exposures and exposure-response patterns. This will enable scientifically-based decisions by national, EU and international bodies on preventive actions.

x) We will explore mechanisms of uptake and distribution of toxic and essential elements in plants, which will make it possible to breed species with low concentrations of toxic elements and high of essential. This gives a possibility to change the intakes through plant foods and the transportation into animal foods.

All these issues have been addressed by the Project and conclusive or at least indicative responses have been arrived at, as shown below.

What are the problems?

Pillar I Nervous system effects/disease

It has been known for long that metals like lead and methylmercury are neurotoxic, particularly at early-life exposure. However, the exposure-response relationships at low exposure have been a matter of debate. Three of the PHIME WPs concerned early-life neurotoxicity of methylmercury, focusing on maternal exposure via fish consumption during pregnancy, and with focus on interactions with nutritional and genetic factors. Another WP had a similar focus for lead-exposed children. Other potentially neurotoxic metals, such as arsenic, cadmium and manganese, which previously had rarely been studied, were covered in two WPs.

The projects have considerably increased our understanding of early-life neurotoxicity of metal exposure through food and drinking water. Major new findings concern the importance of evaluating interactions between neurotoxic agents and nutritional factors for assessment of risk of neurotoxicity. Thus, the toxicity of methylmercury in fish is partly masked by the concurrent intake of long-chain n-3 polyunsaturated fatty acids (PUFAs) from the fish.

Other new findings were that prenatal arsenic exposure through drinking water and food (mainly rice) may cause impaired fetal growth and immunosuppression (developmental immunotoxicity), thus increasing the risk for infant morbidity and impaired neurodevelopment. The latter was observed first at school-age. Further, PHIME studies showed, for the first time, adverse effects on early-life development of prenatal exposure to cadmium through maternal intake of cereals and vegetables. In addition, effects on the central nervous system of very low early-life lead-exposure were shown.

Industrial manganese contamination caused increased concentrations in the part of deciduous teeth corresponding to prenatal exposure, which opens a new tool for biomonitoring. Manganese exposure through vegetables contaminated through soil seemed to be associated with central nervous effects in children (as well as adolescents and elderly in the same area).

One WP concerned methylmercury exposure, both in early and later life, and Parkinson´s disease. Subjects heavily exposed to methylmercury and persistent organochlorine pollutants (POPs) had an increased risk. Exposure to elemental mercury vapour in connection with burning of gold amalgam in gold mining was shown to be high, and responsible for neurotoxicity.

Importantly, several of the studies identified major genetic influence on the metabolism and toxicity of metals. Thus, arsenic detoxification (toxicokinetics) was profoundly influenced, as was the toxicity (toxicodynamics). Further, the metabolism of and neurotoxicity of lead was modified by genetic traits in children and lead workers (Pillar III). Also, the toxicokinetics of both elemental (inorganic) and methylmercury were modified by several genotypes.

Indeed, the toxic metals are very useful tools when assessing gene-environment interactions generally, because it is rather easy to find large exposed populations and to ssess their exposure by biomarkers. Also, toxic effects can be measured accurately.

Hence, the studies within PHIME on effects of metals on the nervous system have generated a wealth of new information, which will be useful in risk assessments and prevention of human exposure, in Europe and elsewhere. Further studies should focus on the consequences of combined exposure to the neurotoxic metals, which are all present in common foods.

Pillar II Cardio- and cerebrovascular diseases, kidney and bone effects

Sub-Pillar II:1 Mercury and cardio- and cerebrovascular diseases

Mercury is toxic to the cardiovascular system, an effect that could start already from fetal exposure. Three PHIME WPs addressed this. In the Faroe Islands, a cohort has been recruited and exposures to methylmercury, POPs and nutritional factors have been determined. Data analysis is ongoing.

In nested case-referent studies by use of a very large biobank containing population-based samples from previous, population-based health-screenings of northern Sweden subjects, no harmful associations were found between methylmercury in erythrocytes and risk of stroke or myocardial infarction (MI); a seemingly protective association for MI is probably because the biomarker is a good marker of fish consumption. No significant genetic modifying effects of glutathione-related genes were seen for methylemercury exposure and MI risk, but there was genetic modification of the toxicokinetics of methylmercury.

In a large pooled study, with male MI cases and controls from Finland and Sweden, advanced probabilistic modelling showed that PUFAs modified a detrimental effect of mercury exposure on MI risk. This study is a very good illustration of a risk-benefit pattern, in the same way as mentioned above for effects of methylmercury and PUFAs on the central nervous system.

A whole-genome single-nucleotide polymorphism (SNP) association study was performed in Eastern Finnish and Northern Swedish MI cases and controls. Top hits included SNPs from genes involved in energy and structural metabolism, as well as genes encoding various types of regulative proteins. Genomic analyses show differences between the Swedish and Finnish populations in general, and in mercury-accumulation related SNPs. Also, the strongest MI genes differ.

In summary, the results indicate that high exposure to methylmercury through fish is a risk factor for MI, but the risk is modified by the simultaneous intake of PUFAs, which counteracts the methylmercury effect. Hence, the net risk is dependent upon the relative levels of methylmercury and PUFAs in the fish, and the amounts of fish consumed. Thus, the risk patterns vary with fish species, and between individuals and populations consuming varying amounts of fish, possibly also by the genetic traits of the population. Hence, the preventive actions (limits and advice) should vary in different countries.

Sub-Pillar II:2 Cadmium and osteoporosis/fractures

It is well known that high exposure to cadmium causes bone toxicity. However, the consequences of long-term, low-level exposure is much less known. Using data from several different cohorts from Sweden and China, PHIME WPs evaluated the associations between cadmium exposure and bone health. Cadmium exposure was assessed by analysis of blood and urine or dietary intake.

Altogether, the Swedish results provide important support for cadmium exposure at the low levels found in the general European population, being associated with negative effects on bone, as shown by decreased bone mineral density, and increased risk of osteoporosis and osteoporotic fractures. Tobacco smoking is a major source of cadmium, but at the same time it may be a confounder via other toxic components in the smoke. However, the risk tended to be even more pronounced among women who never smoked. The effect of cadmium on the skeleton was, to some extent, counteracted by adequate intake of vitamin A. A Chinese study showed that the bone effects are reversible only to a very limited extent after a reduction of cadmium exposure.

These findings are of high public health relevance, since the main dietary cadmium exposure is *via* whole grains and vegetables, i.e. very important foods, and since osteoporosis and osteoporotic fractures are prevalent disorders. Thus, urgent actions must be taken to reduce the cadmium exposure in the population. Therefore, the pollution of agricultural soil with cadmium must be reduced; the levels already present in soil are often so high that they constitute a health risk though contamination of the crop. Unfortunately, any effect of decreased cadmium pollution will result in a reduction of the exposure only after a long time, but that must not be taken as an argument for continued pollution.

Sub-Pillar II:3 Kidney diseases and diabetes

Several elements of the periodic table are toxic to the kidneys. However, many aspects are not sufficiently well known, or open to discussion. Hence, PHIME WPs studied markers of tubular and glomerular damage, as well as end-stage renal disease.

Studies in Belgian adolescents confirmed the association between low-level urinary excretion of cadmium and lead, and that of low-molecular weight proteins, which are supposed to indicate impaired tubular function. However, the data suggest that the excretion of proteins may in itself contribute to increased concentrations of metals. Therefore, the relationships between cadmium and proteins in urine at low exposure might not indicate renal impairment, but be due to reverse causality. Thus, other effects of cadmium exposure (bone effects and cancer) should also be considered in cadmium risk assessment.

In Bangladesh, there were marked differences in metal exposure and trace-element intake (urine concentrations of arsenic, calcium, cadmium, iron, mercury, magnesium, manganese, molybdenum, lead, selenium, tin and uranium) by gender, age, tobacco use and nutritional status.

In Morocco high concentrations of the nephrotoxic mycotoxin Ochratoxin A were found in bread, with several observations above the EU limit (3 ng/g). Despite this, there were no associations between Ochratoxin A in serum and kidney-effect markers in women, and no interaction with cadmium.

In China, reexamination of a population after reduction of cadmium exposure showed that cadmium-induced albuminuria is reversible, while for tubular effects (at considerably higher levels than in the Belgian adolescents mentioned above), there were only slight tendencies towards improvement, but not complete recovery. Gene-environment interaction for cadmium nephrotoxicity was suggested, which may be important from a risk assessment point of view, since genetics may cause variation in susceptibility between individuals and populations.

In a Swedish prospective case-control study, by use of biobanked blood, donated at a health examination, preliminary data indicate that low exposures to lead - and perhaps cadmium - predict future risk of developing end-stage renal disease (uremia), suggesting that these metals may contribute to the development of the disease in the general population. This is probably the first prospective study at low-level exposures.

In a corresponding study of diabetes mellitus, the over-all risk, according to preliminary analyses, was not associated with erythrocyte cadmium, but in sex-specific analyses, a tendency appeared for men, who may be sensitive.

In conclusion, while doubt is casted on the causality of associations between markers of renal tubular effects and low-level metals excretion, the use of sample biobanks now facilitates prospective studies of long-term, clinically important kidney effects, and the first such study suggests an effect of lead - and maybe also cadmium - at low levels. The importance of a long-term view on kidney effects is further stressed by the study of previously highly exposed individuals, showing no or only limited recovery from tubular kidney effects.

Pillar III Where are the problems?

In order to evaluate the public-health consequences of long-term, low-level exposure to metals, there is a need for new information, both on toxic effects (Pillars I and II) and information on the exposures in general populations. Pollution by heavy metals varies between different European and other areas, and it changes over time.

International comparisons of metal exposure in children and women

Blood from 1,363 children from six European (Croatia, Czech Republic, Poland, Slovakia, Slovenia, and Sweden) and three non-European countries (China, Ecuador, and Morocco) showed remarkably small differences between the European cities (the geometric means ranged 0.11-0.17 µg/L for cadmium and 14-20 µg/L for lead). The European differences were also small among 480 women (0.25-0.65 and 14-27 µg/L, respectively). Mercury varied between countries, probably because of differences in tooth restoration practice and fish-consumption habits. In the non-European countries, the levels were higher. As regards industrially polluted areas, the results clearly showed that children living in certain such areas in Europe may have cadmium and lead levels in blood that are about double those in less polluted regions.

Women's blood samples were analysed for platinum, palladium and rhodium, elements which are emitted from automotive catalytic converters, by the best technique. The results indicate extremely low concentrations, in the order of single ng/L, much lower than previously reported.

An important experience was that in international comparisons, there is a strong need for very careful study designs, in particular with regard to analytical performance.

Time series in females in northern and children in southern Sweden suggest a continuation of the decrease in blood lead, which started soon after the decrease of pollution by lead from petrol. For women and children there is also decay for mercury. However, no decrease has been seen for cadmium; thus, there is a need for actions against cadmium pollution, in particular because of the above-mentioned effects on bone, even at "low" exposure. Time trend data from Poland, Czech Republic and Slovakia are under processing.

Biomonitoring of lead exposure

Blood-lead level is the most widely used of all biomarkers of exposure and risk. However, it has limitations, since there is a saturation phenomenon at high exposure. Hence, one PHIME WP, on Swedish and Chinese lead workers, examined whether lead concentration in blood plasma would be a useful alternative. In exposed workers, plasma lead had an acceptable performance, and displayed a higher ratio between exposed *versus* unexposed individuals than either blood or urinary lead, and also showed closer correlations with effects on peripheral nervous system, kidney function and haem synthesis. However, at the low levels found in the general population, the variation in plasma lead data is too large to make it a useful marker.

The effects of lead on the peripheral nervous system, kidney function and haem synthesis in Chinese lead workers were modified by genetic polymorphism in the δ-aminolevulinic acid dehydratase (*ALAD*) and vitamin D receptor (*VDR*) genes, which may explain some of the differences in vulnerability between individuals and populations (see also above, Pillar I). Data on lead workers from Poland and the Czech Republic are presently analysed.

Pillar IV Possible solutions to the problems

Zinc biofortification of cereals: problems and solutions

The goal of biofortification is to develop plants that have an increased content of bioavailable nutrients in their edible parts. Cereals serve as the main staple food for a large proportion of the world population, but have the shortcoming, from a nutrition perspective, of being low in zinc and other essential nutrients. An important finding is that major bottlenecks in plant biofortification appear to be the root-shoot barrier and - in cereals - the process of grain filling. PHIME results demonstrate that the root-shoot distribution of zinc is controlled mainly by heavy-metal transporting P1B-ATPases and the metal-tolerance protein (MTP) family. A greater understanding of zinc transport was important to improve crop quality and also to help alleviate accumulation of any toxic metal. The major molecular results of the work are summarized in **Figures 1** and **2**.

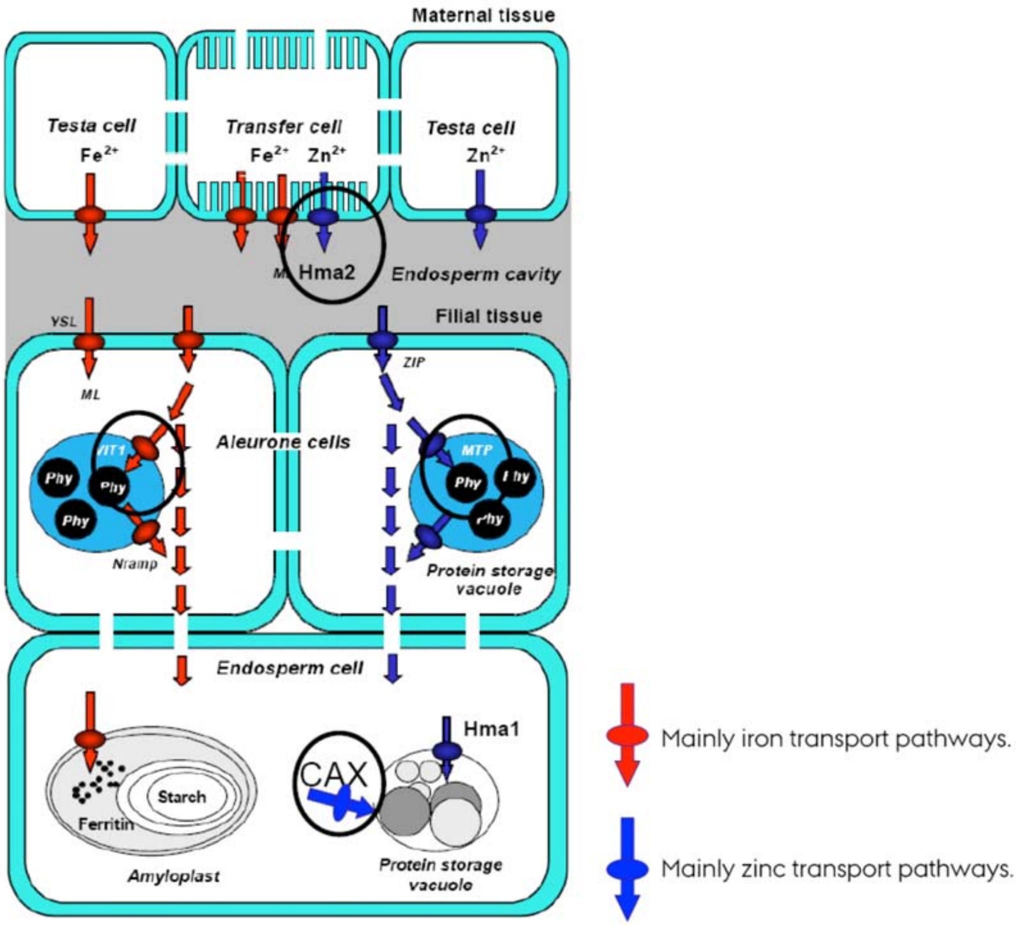

Figure 1. Transport proteins identified with a likely role in heavy metal loading into the cereal grain. Phy: phytate; ML: Metal ligands like metallothionein, citrate or nicotianamine; VIT: Vacuolar iron transporter; Nramp: Iron efflux transporter; MTP: Metal Tolerance Protein; ZIP: Zinc Influx Transporter; YSL: Yellow Stripe1-like Transporter.

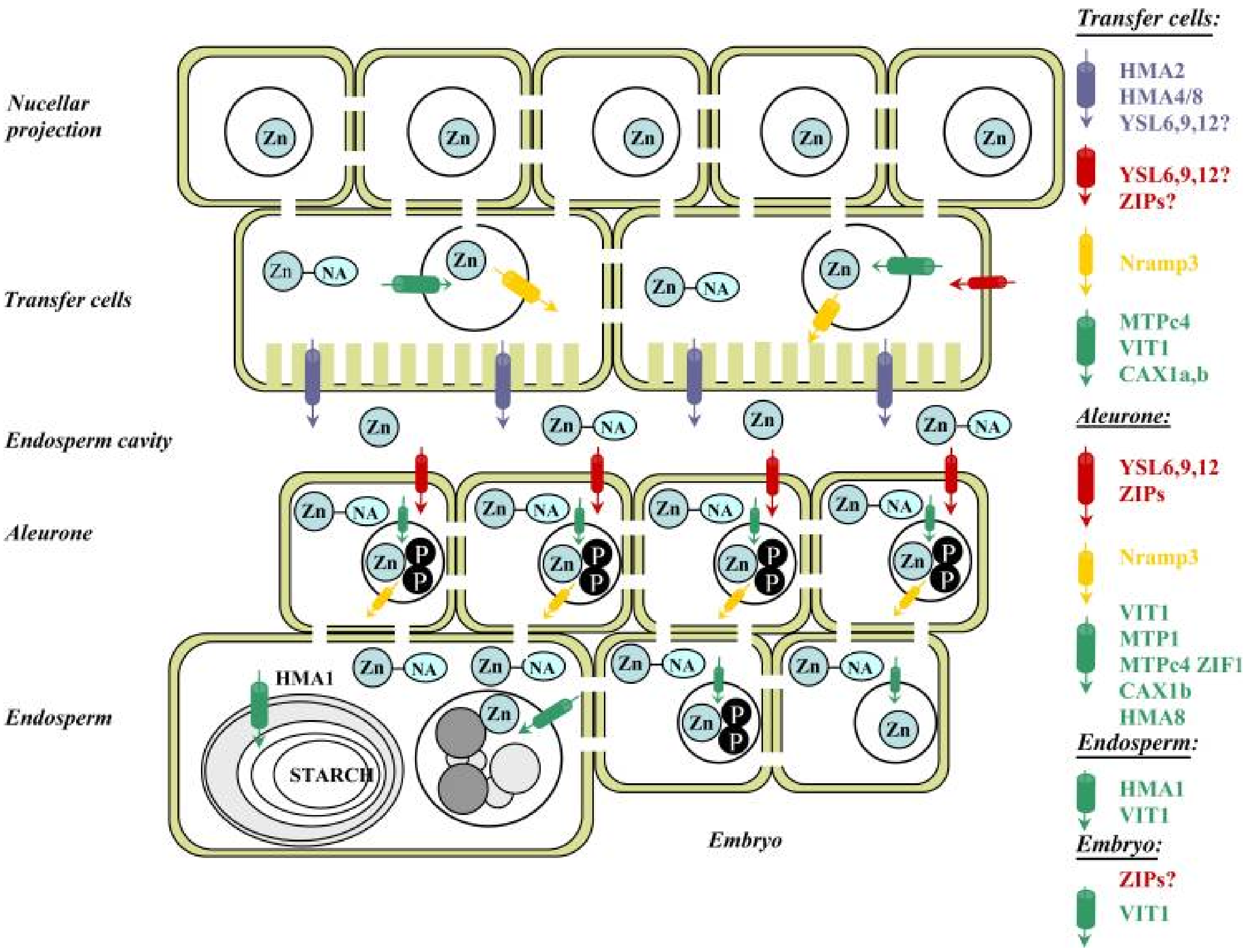

Figure 2. Proposed road map for zinc trafficking from the phloem to the final sequestration sites in the developing grain. Blue cylinders indicate cellular efflux transporters, red cylinders indicate cellular importing transporters, green cylinders indicate vacuolar uptake transporters, and yellow cylinders indicate vacuolar efflux transporters: Zn, zinc; NA, nicotianamine; P, phytate.

A breeding approach to produce nutritionally improved cereals relies on genetic diversity in natural populations that could be crossbred to introduce traits/genes from one variety or line into a new genetic background. There is limited documentation for the natural diversity in grain zinc content of cultivated wheat or rice varieties. More primitive varieties, however, could be a valuable genetic resource. *Gpc-B1* (Grain Protein Content B1) is a wheat quantitative trait locus associated with increased grain protein, zinc and iron content. Introduction of the *Gpc-B1* locus from *Triticum turgidum* ssp. *Dicoccoides* into different recombinant chromosome substitution lines results in higher concentrations of zinc, iron, manganese and protein in the grain, compared to the concentrations in lines carrying the allele from cultivated wheat.

Increased metal content is associated with earlier senescence, but it remains to be investigated whether the introduction of the *Gpc-B1* locus influences grain yield negatively. Lower yields are not likely to be an attractive solution to the micronutrient problem for farmers and for the world's population. Moreover, lower yields would imply that total amounts of micronutrients loaded into the grains of *Gpc-B1* wheat are not enhanced. Information on other loci influencing micronutrient grain content is scarce. To learn more about the genetic basis for cereal grain metal contents, an obvious approach was to analyse genetic diversity in model cereals such as rice and barley.

A transgenic approach to increase the zinc content of cereal grains might involve the manipulation of transporters involved in zinc translocation. With respect to zinc uptake, translocation and deposition, a predominant role seems to be played by members of the ZIP family and, at least in dicots, the MTP family. Furthermore, with respect to root-to-shoot allocation of zinc, the pump HMA4 seems to be a major player in dicots. It remains to be tested whether HMA4 might be used for transgenic biofortification approaches in cereals. To eliminate the need for transgenes, it might be possible to achieve the same results by targeted molecular breeding. A more conventional approach would be to increase the use of zinc fertilizers (fortification) to overcome the problems of zinc deficiency. However, although application of zinc fertilizers should potentially reduce grain cadmium concentration, this depends on the concentrations of cadmium in fertilizers, which indeed remain a major source of cadmium taken up by crops.

Section 1 Project Execution

Section 1.1
What are the problems?
Pillar I Nervous system effects/diseases

This report includes many new, very important results concerning neurotoxicity of metals, which are also reflected in the publication lists. Most of the new results concern early-life exposure and gene-environment interaction, but there are also studies of effects on the adult nervous system. Some additional aspects of neurotoxicity of lead in children are reported under Pillar III below (WP III:2).

Overwiew of Pillar I projects (Tables 1-5)

Table 1. Overview of Pillar I studies.

Cohort (WP)	**Study area**	**Target population**	**Recruit-ment time**	**Inclusion year**	**N**	**Age at outcome test**
Faroe Islands 1 (WP I:1)	Faroe Islands	Mother – Child Infants	At birth	Started	500	2 weeks
Seychelles (WP I:2)	Mahe Island	Children Mother – Child	Early pregnancy	2001 1989	229 550	5 yrs 19-20 yrs
Mediterranean (WP I:3)	Gulf of Trieste; Slovenia; Croatia; Eastern Aegean islands	Mother – Child	Pregnancy	2006-2010	2,189	18 m, 6 yrs
Bangladesh (WP I:4)	Rural area SE Dhaka	Mother – Child	Early pregnancy	2001-2003	2,500	Birth, 7 m, 18 m, 5 yrs
Faroe Islands 2 (WP I:5)	Faroe Islands	Parkinson´s disease	Case-control	2005-2007	172 + 1,018	Adults
Italy (WP I:6)	Brescia region	Workers Children Elderly Pregnant women	Cross-sectional	2007-2008	83 311 255 50	Adults 11-13 yrs 65-75 yrs 25-35 yrs
Poland (WP I:7)	Sosnowiec	Children	Cross-sectional	1997-2003 2007	231 301	8 yrs
Ecuador And other countries (WP I:8)	Ecuadorian Andes Indonesia Tanzania Zimbabwe Philippines	Gold miners and controls Gold miners, inhabitants in gold-mining and control areas	Cross-sectional	2007-8 2010	237 + 72 1,017	Adults and children

Table 2. Main contaminants investigated in Pillar I studies.

Cohort Contaminant	Faroe Islands 1	Sey-chelles	Mediter-ranean	Bangla-desh	Faroe Islands 2	Italy	Poland	Ecuador Others
MeHg	X	X	X	X	X			
Inorganic Hg			X					X
Arsenic				X		X		
Cadmium			X	X			X	
Manganese				X		X		
Lead			X	X	X	X	X	
POPs*	X		X	X	X	X[1)]		
Others			Ni					

* Persistent organochlorine pollutants
1) Pending additional funding.

Table 3. Main outcomes investigated

Cohort / Outcome	Faroe Islands 1	Seychelles	Mediter-ranean	Bangla-desh	Faroe Islands 2	Italy	Poland	Ecuador Others
Anthropometry	X	X	X	X	X	X	X	X
Neurological/ development	X	X	X	X	X	X	X	X
Immune function				X				
Nutrition	X	X	X	X		X		
Parkinson					X	X		
Oxidative stress				X		X[1]	X	X
Genetic polymorphism		X	X	X		X	X	X
Endocrine markers						X[1)]		

1) Pending additional funding.

Table 4. Main nutritional factors assessed by biomarkers in Pillar I studies.

Matrix \ Cohort	Faroe Islands 1	Seychelles	Mediterra-nean	Bangladesh	Faroe Islands 2	Italy	Poland	Ecuador Others
Folate				X				
Fatty acids		X						
Choline		X						
Zinc			X	X		X[1]		
Selenium	X		X	X	X	X[1]	X	
Copper				X		X[1]		
Iron		X	X			X		
Ferritin		X		X		X	X	
Hemoglobin	X	X		X		X	X	X

Table 5. Samples collected in Pillar I studies.

Matrix \ Cohort	Faroe Isl. 1	Seychelles	Mediterra-nean	Bangladesh	Faroe Islands 2	Italy	Poland	Ecuador Others
Drinking water				X		X		
Air particles/dust						X		
Food		X		X		X		
Blood	X	X	X	X	X	X	X	X
Urine			X	X		X	X	X
Hair	X	X	X		X	X		
Placenta				X		X		
Breast milk		X	X	X		X		
Cord blood	X	X	X	X		X		
Umbilical cord	X							
Fingernails						X		
Deciduous teeth						X		

WP I:1 Effects of exposure to methylmercury on the fetal brain: The Faroe Islands study

Summary description of WP objectives

Previous birth cohorts established in the Faroe Islands were extended by a new, prospective recruitment of 500 mother-child pairs. Data on obstetrical history, dietary information and exposure data was collected from umbilical cord blood, and maternal blood and hair. Two weeks *post partum*, a neurological examination was conducted by a pediatrician.

Contractors involved

SDU, FHS (WP leader Pál Weihe)

Degree to which the objectives were reached
The objectives were reached by the establishment of the cohort of 490 mother-child pairs (the intended number was 500). All relevant obstetrical data are collected, mercury has been measured in umbilical cord blood, maternal blood and hair, selenium in maternal blood, and persistent organochlorine pollutants (POPs) in maternal serum. However, the analyses of fatty acids are not completed. All data are digitally available - obstetrical data, dietary questionnaire and the neurological optimality score (NOS).

Among preliminary data, whole-blood mercury concentrations in maternal and cord blood correlated well, but this correlation was substantially improved when adjusting for the haemoglobin concentration. This finding therefore suggests that the precision of this exposure biomarker will be improved by adjustment for haemoglobin. In addition, statistical models have been developed to determine whether the selenium concentration affects the transfer of methylmercury across the placenta. No such interaction could be found, and models with selenium did not offer any advantage over models without.

There is a significant difference in mean NOS at age 2 weeks between first and third ($p=0.041$), as well as between first and fourth ($p=0.028$) quartile groups (cord-blood mercury of first quartile group: <3 μg/L; second: 3-5 μg/L; third: 5-7 μg/L; fourth >7 μg/L). Why, contrary to hypotheis, NOS gets higher with *higher* exposure for mercury is not clear (perhaps the benefits of marine food are more important at the generally low mercury levels, so they can counteract the negative effects of methylmercury).

This means that the cohort formation is completed and ready for follow-up. This has, in fact, already been done at the age of 18 months – with funding from the Danish Ministry of Research. A neuropsychological status is intended at the age of 60 months, if funding is obtained.

Methodologies and approaches employed
The methods used in the recruitment of mothers to the cohort proved to be effective, since the participation rate is acceptably high (73%). Exposure assessment using whole blood from the cord proved successful, and adjustment for haemoglobin showed a substantial advantage that improved the correlation between mercury concentrations in maternal blood and cord blood.

The Groningen Neonatal Neurological Examination records systematically main neurological functions, such as posture, motility, movements, tonus, responses, tendon reflexes, other reactions, reactivity, and stability of behavioral status during the examination. The technique for the examination of the children is a comprehensive age-adequate examination as described by Prechtl. [Prechtl HFR: The neurological examination in the full-term newborn infant. 2nd ed. Clinics in Developmental Medicine, No. 63. SIMP. London: Heinemann, 1977.] It has proven to be predictive for later major and minor neurological dysfunction.

Achievements in relation to the state-of-the-art
In a society where four other cohorts of newborns have been established since 1985, the recruitment efforts this time have reached their highest level by using the obtained experience in cohort establishment. The mercury concentrations are much lower now, thus documenting the effect of the food advisories issued by the Faroese health authorities. Methods for exposure assessment have been further improved and validated.

Conclusions
A new cohort is established with an exposure level lower than the four previous cohorts. Eventually this will give us the opportunity to ascertain the dose-response relationship at low doses and the possible interaction with polychlorinated biphenyls (PCBs), the exposure to which has decreased to lesser degree than mercury.

WP I:2 The Seychelles Child Development Studies

Summary description of WP objectives
The aims of the Seychelles Child Development Studies (SCDS) were to investigate the effects of prenatal methylmercury (MeHg) exposure (from fish consumption) in early child and adulthood, and to examine the nutritional factors which may modify these effects. This WP focused on two longitudinal cohorts: (1) children aged 5-6 years (*nutrition cohort*) and (2) adolescents aged 19-20 years (*main cohort*). Specifically, the objectives of this WP were to (a) collect prenatal hair samples from mothers, (b) collect dietary data from children, (c) collect blood samples from children for the analysis of the potential confounding nutrients, long chain polyunsaturated fatty acids (PUFA), choline, betaine and iron, and (d) to perform psychological tests in these cohorts. Postnatal hair samples were also collected from children.

Contractors involved
UUC (WP leader Sean Strain), MOHS, Uroch.

Degree to which the objectives were reached
All objectives were met and all milestones and deliverables were completed on schedule. Psychological testing of a total of 229 children in the nutrition cohort was completed in July 2008 and testing of 532 adolescents in the main cohort was completed in September 2009.

Methodologies and approaches employed
Blood analyses for the cohorts included fatty acid analysis, iron status measurements (nutrition cohort only) and assessment of choline and betaine status. Four day food diaries were collected from the nutrition cohort and food frequency and fish consumption questionnaires were collected from the main cohort. At 5 years of age, children completed a range of psychological tests including Finger Tapping Test, Preschool Language Scale, Woodcock Johnson Tests of Achievement, Achenbach Child Behaviour Checklist (CBCL) and Kaufman Brief Intelligence Test (K-BIT). Testing in the adolescent group involved Finger Tapping Test, K-BIT and Profile Of Mood States (POMS). The effects of prenatal exposure to MeHg and early child and adulthood development were investigated and the role of nutritional factors in the modification of this association was explored using regression models.

Achievements in relation to the state-of-the-art
5 year old children – Nutrition cohort
A total of 300 mothers were enrolled early in pregnancy in 2001 and their children were followed up at 5 years of age. There were 229 mother-child pairs with complete outcome and covariate data for analysis. Mothers consumed an average of 537 g of fish (nine meals containing fish) /week and the average prenatal MeHg exposure from hair samples was 5.7 ppm.[1] Children at aged 5 years reported eating a mean of 42.2 g fish/d (295 g fish/wk); intakes higher than the UK daily fish intake of 20.0 g/d for children aged 4-10 years. Children appeared to meet the WHO dietary recommendations for most nutrients except calcium, iodine, folate and vitamin D. However, these low intakes may be owing to incomplete nutritional databases, particularly for

iodine and vitamin D, rather than represent true deficiencies, especially in this high fish eating population. Indeed, these dietary findings do not reconcile with our previous physiological findings of adequate iodine status in pregnant Seychellois women (unpublished work). Plasma phospholipid fatty acid concentrations were comparable to previous studies in children at a similar age.[2, 3] Fatty fish consumption was a significant positive predictor of EPA status (β=0.18, p=0.02) and there were no significant sex differences in fish intake or fatty acid status in this cohort.

Regression analysis examined the associations between prenatal maternal nutritional status and children's outcome measures, with and without adjustment for prenatal MeHg exposure (**Table 1**). Prenatal long chain n-3 polyunsaturated fatty acids (LC-PUFA) (particularly docosahexaenoic acid=DHA) was a significant positive predictor of Preschool Language Scale outcomes. Although arachidonic acid (AA) was a significant negative predictor of Preschool Language Scale outcomes, total n-6 PUFA was a significant positive predictor of Child K-BIT – Verbal Knowledge, with or without correction for prenatal MeHg. The AA:DHA ratio was a significant negative predictor of Preschool Language Scale outcomes – total and auditory comprehension, only when prenatal MeHg was not included in the model. No adverse associations were observed with prenatal hair MeHg for any outcome and all LC-PUFA associations were stronger when prenatal MeHg exposure was removed from the models. These results confirm previous findings which suggest a possible confounding role of maternal nutrition (particularly, LC-PUFA) in studies which examine associations between prenatal MeHg exposure and developmental outcomes in children. There appear to be long-term beneficial effects of higher maternal prenatal LC-PUFA status on developmental outcomes. Postnatal MeHg was also measured in hair samples collected from the 5 year old children. The analysis of these data to investigate the effects of postnatal MeHg exposure on cognitive development, and including postnatal LC-PUFA data as covariates, was carried out as a secondary analysis. Results showed no effects of postnatal MeHg or postnatal LC-PUFA on any outcome. Prenatal AA was found to remain a significant negative predictor of Preschool Language Scale auditory comprehension, whilst adjusting for postnatal LC-PUFA but without adjusting for prenatal and postnatal MeHg (p=0.021). The effect almost reached statistical significance when MeHg (prenatal and postnatal) values were adjusted for in the model (p=0.051).

Table 1. Regression analysis investigating associations between prenatal maternal nutritional status and 5 year old children's developmental outcomes (with and without adjustment for prenatal MeHg)*

	With MeHg	Direction of effect	P value	Without MeHg	Direction of effect	P value
Model 1 - DHA, AA						
Preschool language scale – total language raw score	DHA AA	20.094 -8.293	0.034 0.016	DHA AA	24.370 -9.085	0.006 0.007
Preschool language scale – auditory comprehension raw score	AA	-3.930	0.022	DHA AA	10.270 -4.329	0.021 0.011
Preschool language scale – verbal knowledge raw score	DHA AA	11.981 -4.363	0.045 0.043	DHA AA	14.104 -4.757	0.012 0.025
Model 3 – n-3, n-6						
Child K-BIT – verbal knowledge	n-6	0.345	0.020	n-6	0.344	0.020
Model 4 – AA:DHA ratio						
Preschool language scale – total language raw score	AA:DHA ratio	NS		AA:DHA	-0.861	0.027
Preschool language scale – auditory comprehension raw score	AA:DHA ratio	NS		AA:DHA	-0.387	0.047

*all models adjusted for sex, family status, maternal age, maternal IQ (K-BIT), SES (Hollingshead), Home environment (PROCESS), child's age and child's birth weight, NS not significant

19-20 year adolescents - Main cohort

A total of 779 mothers were enrolled early in pregnancy in 1989 and their children were followed up at 19-20 years of age. A total of 532 adolescents were assessed. Mothers consumed an average of 12 fish meals per week and the average prenatal MeHg exposure from maternal hair samples was 5.9 ppm.[4] Adolescents at 19 years consumed fish on average 7.4 times/wk (range 0-29 times/wk), with males eating significantly more fish in a week compared to females (mean 8.0 -*vs*- 6.9 fish /wk). Median energy intakes of both male and female adolescents were low compared to WHO recommendations, albeit intakes of macronutrients were adequate. Suboptimal intakes of potassium, calcium, magnesium, copper, selenium, iodine and vitamin D were apparent for both males and females. Again, these low intakes may be owing to incomplete nutritional databases rather than representing nutritional deficiencies. In addition, iron intakes were low in females, as is common in this age group.[5] Plasma phospholipid fatty acids are similar to those reported by other studies of healthy adults.[6, 7] There were no significant differences in fatty acid status between males and females.

Prenatal MeHg exposure did not have any detrimental effect on developmental outcomes at 19 years of age. Postnatal MeHg was a significant negative predictor of K-BIT Matrices standard score with and without adjustment for postnatal LC-PUFA (**Table 2**). Postnatal MeHg was also a significant negative predictor of finger tapping non-dominant hand in females only, with and without adjustment for postnatal LC-PUFA (p=0.004 for both models). These results confirm previous findings from the main cohort which indicate that prenatal MeHg exposure does not have any effect on developmental outcomes from infancy through to 19 years of age.

Age and sex differences in plasma total homocysteine (tHcy) concentrations and related metabolites were evident within and between the two cohorts. Plasma tHcy and methionine concentrations were significantly higher and choline and betaine concentrations were significantly lower in adolescents compared to 5 year old children. Males had significantly higher concentrations of all metabolites than females; albeit these sex differences were only evident in the older age group, concurring with previous studies.[8, 9]

Table 2. Regression analysis investigating associations between prenatal and postnatal Hg and 19 year old developmental outcomes (with and without adjustment for postnatal LCPUFA)*

	With LCPUFA	Direction of effect	P value	Without LCPUFA	Direction of effect	P value
KBIT Matrices	Postnatal MeHg	-0.379	0.020	Postnatal MeHg	-0.390	0.012
Finger-tapping (non-dominant hand) FEMALES ONLY	Postnatal MeHg	-0.250	0.004	Postnatal MeHg	-0.229	0.004

*all models adjusted for sex, maternal IQ (K-BIT) and SES (Hollingshead)

Conclusions

These results are confirmative of previous findings from the SCDS and which suggest a confounding role of maternal nutrition in studies examining associations between prenatal and postnatal MeHg exposures and developmental outcomes in children. LC-PUFA present in fish and in the overall diet can obscure the determination of adverse effects of prenatal MeHg exposure in longitudinal observational studies.

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WP I:3 Child neurodevelopment among residents in the Mediterranean coastal regions of Italy, Slovenia, Croatia and Greece: the role of environmental exposure to heavy metals

Summary description of WP objectives

Mercury (Hg) is an element that is ubiquitous in nature and is methylated by bacteria in aquatic environments to form methylmercury (MeHg). Concentration in the food chain may lead to high levels in predatory fish and sea mammals. MeHg is a neurotoxicants, with children and developing fetuses being the most susceptible to these health effects. Primary human exposure occurs through intake of contaminated food, namely seafood in the diet. There have been several poisoning episodes of MeHg, which have led to a greater understanding of the effects of MeHg on the human nervous system and - more importantly - the effects on the developing nervous system. The basis for the research rests in the fact that there is still a great deal of controversy over the following issues:

- Whether or not prenatal and postnatal exposure MeHg or inorganic Hg actually leads to health effects in children at the low doses consumed in fish-eating European populations
- The role on neurodevelopment of other potential factors in the diet, such as other metals and elements, fatty acids and additional pollutants is poorly understood.

To evaluate the impact of Hg exposure through food consumption on the developing nervous system among residents in Italy, Slovenia, Croatia and Greece, we conducted a prospective cohort study within the WP, which also considered various other demographic, nutritional and toxic environmental factors.

Contractors involved
UNIUD (WP leader Fabio Barbone), IJS, Oikon, UMCL.

Degree to which the objectives were reached
As planned, 2,189 mothers were enrolled in the study during pregnancy. 1,729 mother-child pairs provided information at child's birth. Neurodevelopmental outcomes were measured in 1,439 children, i.e., the follow-up rate at 18 months was 83%.

Methodologies and approaches employed
The following table describes the study instruments.

	Enrolment Brief Questionnaire Hair sample collection	Prenatal Maternal Blood and Urine Sample Collection	Long Questionnaire	Postnatal Sample collection	Outcome assessment	Other measures, questionnaires
UNIUD (Italy)	20-22 weeks EGA	20-32 weeks EGA	28-32 weeks EGA – 1 month post delivery	Cord blood, at delivery. Maternal hair, breastmilk at 1 month post delivery Child hair at 18 months.	18 months BSID 3rd	Raven to the mothers in pregnancy. Supplementary Questionnaire at 18 months.
IJS (Slovenia)	Perinatal period (27-32 weeks EGA)	Perinatal period	1 month post delivery	Maternal hair, cord blood at delivery. Breastmilk at 1 month post delivery.	18 months BSID 3rd	Supplementary Questionnaire at 18 months.
OIKON (Croatia)	34-38 weeks EGA / Perinatal period	34-38 weeks EGA / Perinatal period	1 month post delivery	Cord blood, child's urine or meconium at delivery. Breastmilk at 1 month post delivery.	18 months BSID 3rd	Raven to the mothers perinatally. Supplementary Questionnaire at 18 months.
ChildH (Greece)	Perinatal period	No	3-6 months post delivery	Maternal hair, cord blood, maternal and child's urine or meconium at delivery. Rarely breastmilk at 3-6 months post delivery	18 months BSID 3rd	Supplementary Questionnaire at 18 months.

The statistical analysis included:
- Descriptive analysis: distribution of all the variables (mean, SD, quartiles, min-max range for continuous variables; frequencies for categorical ones);
- Tests for normality (Shapiro-Wilk) for continuous variables, transformations;
- Bivariate analyses: correlations, Wilcoxon's rank sums tests, Kruskal-Wallis tests;
- Analysis of missing values and inputations where appropriate for multivariate analyses;
- Multivariate analysis: linear regressions.

- Main exposure estimate was total Hg (THg) concentration in mother's hair in pregnancy and in cord blood, and mother's fish intake in pregnancy.
- Child's neurodevelopmental outcome was the Bayley Scales of Infant and Toddler Development (BSID III) for the three major domains (cognitive, language and motor) at 18 months.
- A number of covariates were evaluated in multivariate linear regression models:
  - Pregnancy history (mother's age at delivery – mother's IQ in Italy and Croatia - body mass index (BMI) before pregnancy - weight gain increase - cigarettes smoked throughout the pregnancy - weekly alcohol intake - dental visits and new/replaced dental fillings);
  - Socioeconomic status (SES; home size - home ownership - parental education - number of children in the family - marital status of the mother at delivery);
  - Child (sex – birth weight - breastfeeding history - daycare attendance at 18 months - duration of the intake of fresh and homogenized fish up to 18 months);
  - Setting: country - time of Bayley test.

A complete maternal and child diet was also evaluated, maternal polyunsaturated fatty acids (PUFAs) were measured in Italy and Croatia, and the concentrations of Se, Pb, Zn, Cu, Mn, As in venous and cord blood, urine, and milk were measured in all countries.

The possible interactions between genetic polymorphisms and Hg concentrations, fish intake and PUFAs levels are being evaluated. Extensive genotyping has been performed.

The following table describes the achievements of the data collection by country:

	Country			
Database	**ITALY**	**SLOVENIA**	**CROATIA**	**GREECE**
Biological samples	900	656	234	484
BriefQ	900	591	234	469
Raven	900	-	130	-
LongQ	767	373	200	391
Bayley	632	286	161	350
SupplementaryQ	522	200	73	290
Used in analysis of association Hg-neurodevelopment presented today (pre-term births, twins, and Bayley done at ≥20 months excluded)	525	210	111	302

The median of mother's hair THg was 729 ng/g. Mean ratio MeHg/THg was 0.90. Median cord blood THg was 3.75 μg/L. Correlation between THg in hair and cord blood was high, $r=0.81$. Median weekly servings of fish was 1.38.

While unadjusted models showed a negative association of THg (in both hair and cord blood) with Bayley cognitive scores, after adjustment for country and relevant covariates, Bayley cognitive score was not associated with hair THg ($\beta=0.00026$; $p=0.5157$), nor with cord-blood THg (0.048; $p=0.6318$), but positively associated with fish ($\beta=0.8345$, $p=0.0162$). Bayley language score was not associated with cord blood THg ($\beta=0.0412$, $p=0.7037$), borderline positively associated with hair THg ($\beta=0.0008$, $p=0.0552$), and significantly positively associated with fish consumption in both unadjusted and adjusted models ($\beta=0.8564$, $p=0.0083$).

Bayley motor score was negatively associated with hair THg in unadjusted models (β= - 0.00073, p=0.0022), but not in adjusted models (hair THg: β= - 0.00017, p=0.553; cord blood: β=0.0038, p=0.9583).

Achievements in relation to the state-of-the-art

This Mediterranean, multicenter epidemiologic study is the largest ever conducted in the general European population on the impact of mercury exposure through food consumption on the developing nervous system as it is evaluated prospectively on a mother-child cohort.
Through this research, we provide evidence that, overall, exposure to Hg is low in this Mediterranean population and at these levels do not significantly affect neurodevelopment by age 18 months. Exposure to higher Hg levels during pregnancy did not cause lower performances in cognitive, language and motor neurodevelopmental testing, since other correlated variables, such as country of residence, socioeconomic indicators, maternal IQ and other non-environmental factors, predict these outcomes. Rather, higher fish consumption in pregnancy was associated with higher cognitive and language (but not motor) neurodevelopmental performance at 18 months of age.

Preliminary results of this investigation were presented orally at the two largest conferences on the topic 2011, i.e., the 10th International Conference on Mercury as a Global Pollutant (ICMGP), Halifax, July 24-29 2011 and the Conference of the International Society for Environmental Epidemiology (Barcelona, September 13-16, 2011).

Conclusions

THg in mother´s hair and in cord blood did not predict Bayley scores but a moderate beneficial effect of fish consumption in pregnancy was observed. Other chemical elements were not associated with the outcomes. Pregnant women should eat fish in pregnancy, choosing from fish species, origin and size associated with lower level of Hg contamination.

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WP I:4 Neurodevelopmental effects of arsenic – interaction with other neurotoxic metals, nutrition and genetic polymorphism

Summary description of WP objectives

The starting point was the prevalent elevated concentrations of highly toxic and carcinogenic inorganic arsenic (As) in drinking water and certain foods world-wide, while very little is known about potential effects of exposure in early childhood. We aimed to elucidate to what extent pre- and/or postnatal exposure affects child development; the major mechanisms of action and potential interactions with other toxic elements, nutrition and genetic polymorphisms.

Partners involved

KI (WP leader Marie Vahter), ICDDR,B, LU.

Degree to which the objectives were reached

All questions raised in our initial proposal were answered. Collected data also enabled exploration of questions beyond the initial proposal, especially regarding early-life exposure to cadmium (Cd). This project has considerably increased the knowledge base on effects of As and Cd on early-life health and development.

Methodologies and approaches employed

Our study is carried out in Matlab, a pristine, rural area in Bangladesh. The main environmental concern is the prevalent elevated As concentrations in drinking water (mainly wells, 70% of which had >10 μg As/L), while environmental pollution, including automobile exhaust, is very low. Our study is nested in a population-based food and micronutrient supplementation trial in pregnancy, including 4,500 women. We have built a longitudinal mother-child cohort with repeated measurements of exposure to As and other potentially toxic metals [mainly Cd, lead (Pb) and manganese (Mn)], as well as repeated measurements of outcomes in more than 2,000 women (recruited in 2002-2003) and their singleton children up to 5 years of age (Vahter et al. 2006; Hamadani et al. 2010).

Exposure was assessed through samples of drinking water, rice, maternal urine in gestational week (gw) 8 and 30 (prior to PHIME), maternal blood (sub-sample) in gw14 and 6 months after delivery, as well as child urine at 1.5 and 5 years of age. In addition, we measured element concentrations in placenta, cord blood, breast milk at 2 months after birth in sub-samples. As, Mn, Cd and Pb were measured by inductively coupled plasma mass spectrometry (ICP-MS), As metabolites by HPLC-ICPMS. Nutrition biomarkers, such as ferritin, zinc (Zn), selenium (Se), Vitamin B12 and folate were measured in plasma of some mothers during and after pregnancy. Data on maternal and child anthropometry and socio-economic factors were obtained.

Child growth and psychomotor development were measured in over 2,000 children at 5 years of age. We assessed development using Wechsler Preschool and Primary Scale of Intelligence (IQ) (data available also for 1.5 years based on Bayley Scales of Infant Development-II). Measured biomarkers of toxic effects include oxidative stress in maternal urine and placenta, immune function and inflammation in placenta and cord blood, as well as genetic polymorphisms of potential importance for As metabolism.

Achievements in relation to the state-of-the-art

It is well known that elevated concentrations of As and Mn in drinking water are common world-wide. This project shows that certain foods, especially rice, are also important sources of As (Khan et al. 2010) and Mn, as well as Cd (Kippler et al. 2010) and Pb (Bergkvist et al. 2010). Besides Pb, there is an almost complete lack of knowledge concerning health effects of early-life exposure of the other toxicants.

Maternal exposure and related effects: Urinary and blood metals concentrations were elevated in the mothers compared to many other populations, As much more so than Mn and Cd. Women with low iron stores had higher uptake of Cd and Mn, indicating increased uptake via intestinal Fe transporters (Kippler et al. 2007 and 2009; Ljung et al. 2009). Exposure to Pb was quite low and seemed to originate mainly from food, soil/dust, and cooking pots (Bergkvist et al. 2010). Oxidative stress marker in the urine in early pregnancy was elevated and related to As, but even more so with Cd (Engström et al., 2010), which has not been shown earlier.

The increased Cd uptake during pregnancy led to high Cd accumulation in the placentas (Kippler et al. 2010). While the placental accumulation of Cd resulted in low cord-blood Cd, it also impaired Zn transfer to the fetus (Kippler et al. 2010a), which seemed to influence fetal growth (Kippler et al. 2011). Unexpectedly, this was mainly seen in girls, not in boys. Maternal As exposure was associated with fewer immune cells and increased markers of inflammation and oxidative stress in placenta and cord blood, indicating immuno-suppression (Ahmed, et al. 2011). There was an unexpectedly small increase in risk of fetal loss and a small effect on size at birth with As exposure (Rahman et al. 2009 and 2010), possibly due to a remarkable increase in As metabolism in the early months of pregnancy (Gardner et al. 2011a). As metabolism appeared to be primarily associated with genetic background, in particular AS3MT polymorphisms (Engström et al. 2010 and 2011). As metabolism was not significantly affected by the women's nutritional status (Li et al. 2008; Gardner et al. 2011a).

Transfer of Cd to breast milk was low, but increased with maternal exposure (Kippler et al. 2009). Probably, Cd is transferred via the active transport system for Fe and Mn. An important new finding was that Cd impaired calcium (Ca) transport to milk (Kippler et al. 2010a). Concentrations of As and Mn in breast milk were very low, and not affected by maternal exposure. Breast-feeding thus limits infant exposure to all the studied elements (Fängström et al. 2008; Ljung et al. 2009).

Child exposure and psychomotor development: Children had wide variation in exposure to As at 1.5 and 5 years of age, although somewhat lower than their mothers (Gardner et al. 2011). Surprisingly, also urinary concentrations of Cd were elevated, particularly at 3 months of age (Kippler et al. 2010b). Evaluation of potential health and developmental consequences of the early-life Cd exposure is on-going. Child urinary Pb (U-Pb) was only slightly elevated. The 1.5 years old children's U-Pb peaked during the monsoon period, probably because of ingestion of soil and mud attached to hands and toys. In contrast, the 5 years old children's U-Pb peaked during the dry season, likely due to inhalation of dust during playing (Bergkvist et al. 2010). Important findings were that Pb was released from cooking pots and tin roofs.

Prenatal As exposure was associated with increased risk of infectious diseases during the first years in life (Rahman et al. 2011), indicating that despite the protective up-regulation of As metabolism in pregnancy, children's immune function may still be impaired by pre-natal As exposure (Ahmed et al. 2011). In terms of motor and cognitive development, we found no significant association between maternal As exposure in pregnancy and child development at 7 or 18 months (Tofail et al. 2009; Hamadani et al. 2010). Most of the infants were breast-fed during the first months, resulting in low As exposure, but exposure through food and water could be considerable in infants who were not exclusively breast-fed (Fangstrom et al. 2008), and in children after weaning (Fangstrom et al. 2009). Preliminary findings from the testing of children at 5 years of age, show significant associations between As exposure and verbal and full scale IQ, but not performance IQ (Hamadani et al. submitted). An important finding was that girls were more affected than boys. It is possible that effects may only become detectable first in later childhood, even if induced earlier in life. Obviously, continuous exposure may aggravate the toxicity.

Conclusions
Extensive pre- and postnatal As exposure through drinking water and food, resulted in increased infant morbidity and mortality, probably largely related to immunosuppression. Also, child cognitive functions were affected. Maternal Cd exposure was somewhat elevated, probably as a result of rice being the main food. This resulted in markedly elevated oxidative stress and impaired micronutrient transport to fetus and breast-fed infants. It is essential to follow-up child health and development in relation to early-life Cd exposure. Also, potential adverse effects of the elevated Mn concentrations in drinking water need to be investigated.

Collaboration with EU NewGeneris project
Initiation of collaboration with the EU project NewGeneris involved measurements of multiple elements in blood samples from the Rhea Cohort in Crete. Smoking was associated with higher Cd concentrations in pregnant women, and we found indications of elevated As and Hg in relation to passive smoking (Vardavas et al. 2011).

Publications
Arsenic

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Cadmium, lead, manganese
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WP I:5 Prenatal methylmercury exposure as a risk factor for Parkinson's disease

Summary description of WP objectives
To assess whether lifetime exposure to contaminants from traditional food, i.e. methylmercury (MeHg) and persistent organochlorine pollutants (POPs), contribute to the increased incidence of Parkinson's disease (PD) in the Faroe Islands.

Contractors involved
SDU (WP leader Philippe Grandjean), FHS

Degree to which the objectives were reached
The objectives have been fully achieved.

Methodologies and approaches employed
Two approaches were used: (1) A study of prenatal exposure to MeHg by use of unique information on intake of pilot whale in different regions of the Faroe Islands. (2) Examination of cases of PD and matched and controls.

PD patients. A total of 102 potential cases of PD were recruited and were clinically examined by a neurologist in 2005. The diagnostic assessment of the idiopathic PD cases was based on clinical information, development of the disease, response to levodopa treatment and used internationally accepted criteria. Cases with parkinsonism, but with additional atypical features, were diagnosed as having other neurodegenerative diseases (Wermuth et al 2008). For the analysis of postnatal MeHg exposures, we included only the 79 cases with idiopathic PD, i.e., 43 males and 36 females. The mean age of cases was 74.4±9.5 years, and the mean age at onset of PD was 65.4±10.7 years.

In regard to assessment of prenatal MeHg exposure, the study was extended with 81 cases, who had been identified and assessed by the same methods in 1995, but who had died before the present study, and 21 PD cases identified in 2005, but who did not actively participate in the study. Thus, all 181 Faroese PD patients (cases) identified in 1995 or diagnosed between 1995 and 2005 were considered for inclusion in this study (Wermuth et al. 2007). Nine PD cases were born before 1912 were excluded, because population numbers at village level were lacking. Hence, a total of 172 PD cases were included.

Controls. We retrieved six controls for each case from the Faroese Population Registry, using vital status in 1995 and 2005, sex and age as matching parameters. Contact was attempted to controls in order to recruit two controls for each case identified in 2005. Potential controls were first invited for study participation by letter and then, one week later, by telephone. From the six potential controls, two were included for each PD case, except for four cases, where only one control had agreed to participate (Halling et al. 2008).

For the remaining controls, information was retrieved from the population registry on place and date of birth only. The birth place of each individual could then be linked to whaling records (see below). A total of 1,032 controls were drawn from the population registry. However, for six controls, information on the birth place was missing and eight subjects were known PD cases, and thus excluded as controls. A total of 1,018 controls were therefore included in the analysis regarding prenatal exposure.

A total of 154 controls were recruited to participate in the postnatal study, i.e. 85 men and 69 women. The mean ages of controls were 75.2±9.6. In the course of recruiting the control group, 77 subjects (49 women and 28 men) declined to participate, and efforts to obtain response from six invited subjects (4 women and 2 men) were unsuccessful. Examinations were carried out at the Department of Occupational Medicine and Public Health in Tórshavn, Faroe Islands; consenting subjects unable to come to the department were visited at home.

Assessment of exposure. Blood and hair samples were obtained from all examined 79 cases and 154 controls and the concentrations of Hg and major POPs were measured. Further, all subjects completed a questionnaire by face-to-face interview, in order to record lifetime information on residence, dietary habits, smoking history, and occupational exposure to solvents, pesticides, and metals.

The prenatal MeHg exposure was determined by an ecological exposure classification from the date and place of birth. The main source of MeHg exposure in this population is pilot-whale meat. The whales are caught by beaching pods that have approached the islands. For each location, the assignment of whale meat per resident was computed from the size of each whale catch, the detailed rules of distribution, and the number of residents entitled to receive a share. Hence, each resident was assumed to receive a share calculated from the total amount of whale meat landed, divided by the number of residents in one or more of the nine major districts of distribution. Utilizing the calculated share that each resident received at the mother's residence prior to the date of parturition, estimation of the maternal body burden profiles could be performed assuming an average MeHg concentration of 2 µg/g whale meat, a regular consumption of the meat at a rate of 3 kg/month, and that any remaining supply after two years would be discarded (for further details, http://www.chef-project.dk/PDF/RetrospectiveExposure_20051221.pdf). Calculations of average hair Hg concentration during the whole pregnancy, during the third trimester, and a point estimate at the beginning of the third trimester (i.e., three months before child birth) were performed, using the individual body-burden profiles for each mother. These numbers were then used to compare estimated prenatal exposures of PD cases with those of the controls.

Statistical analysis. Prenatal study: The Mantel-Haenszel odds ratio (OR) for matched data was used with stratification by age and sex, and MeHg exposure categorised as a binary exposure variable where 'not exposed' was defined as hair-Hg concentration equal to zero or within the lowest tertile of the non-zero hair-Hg distribution, while 'exposed' was defined as hair Hg levels in the second and third tertiles of non-zero hair Hg distribution (Petersen et al. 2008).

Postnatal study: We used multivariate unconditional and conditional logistic regression analyses. We estimated the adjusted OR and 95% confidence interval (CI) after controlling for potential confounding (sex and age, as these factors were matched in the selection of controls) and interaction effects. Further, potential gender differences were examined using stratified analysis techniques (Petersen et al., submitted).

Achievements in relation to state-of-the art

Prenatal study (Petersen et al. 2008)

Thirty controls were born abroad while that was true only for two cases. This difference is not statistically significant (p=0.30). Although not significant, some ORs suggested that subjects with high Hg exposure had slightly greater odds of having PD than those who were not exposed. Overall, no significant association between PD and prenatal MeHg exposure was found in this study. The upper confidence limits were all below 2.0, thus making prenatal MeHg exposure an unlikely cause of the doubled PD incidence in the Faroe Islands. Because these subjects were mostly born in the first half of the 20^{th} century, prenatal POP exposure would not have been of importance.

Postnatal study (Petersen et al. 2008)

ORs for intake of whale meat and blubber in adult life were significantly associated with PD risk. The OR for intake of whale meat in childhood was of same magnitude as in adult life, although not significant. The inclusion of the potential confounders (sex and age) did not materially change the OR estimates. Because of collinearity between blubber and whale meat consumption (r=0.85, p<0.001), it was impossible to separate possible effects of these two parameters. There was no significant difference between cases and controls in regard to consumption of these food items in childhood or during the last year. Gender-stratified analysis revealed higher risks for men, caused by life-time intake of whale blubber and meat compared with women's, but this difference was not significant. In addition, no interaction was found between consumption and residence.

The adjusted ORs suggested an increased risk for PD with occupational exposure to solvents, pesticides and metals, but none of the ORs were statistically significant. For pesticides and metals, the OR is based on the risk in males only, as no female case or control stated exposure to pesticides and only two female controls had experienced exposure to metals. The gender-stratified analysis for solvents showed similar OR in women and men.

The smoking history variable suggested that smoking is associated with lower risk of PD, but the association was not statistically significant. The effect of sex on the OR did not show a uniform, significant tendency.

Both among cases and controls, women had significantly lower serum concentrations than men for all POPs and Hg biomarkers analyzed, except for β-HCH that was significantly higher in women. All log-transformed PCB congeners and other POPs were significantly correlated (p<0.01), with Pearson's correlation coefficients ranging from 0.18 to 0.99. There was a clear effect of age on the current concentrations of ∑PCB, PCB-TEQ (toxic equivalent), HCB, *p,p*-DDE, *o,p*-DDT and β-HCH that also differed between women and men. For β-HCH, sex by itself was not significant. In contrast, age was unrelated to the Hg concentrations, but males had higher levels than females.

The current serum concentration of β-HCH was significantly associated with an increased PD risk. Gender-stratified analysis showed much higher crude OR among women for β-HCH (OR=12.07, 95% CI=2.14-68.18) than men (OR=2.06, 95% CI=0.52-8.15). Overall, the ORs suggest an increased risk for PD with current exposure to ∑PCB, PCB-TEQ, *p,p*-DDE, HCB and blood-Hg, but not for *o,p*-DDT and hair-Hg. Inclusion of possible confounders (sex and age) did not change the OR estimates to any appreciable extent. Adjustment for smoking status did not affect these results. The single PCB congeners showed similar trends with elevated, non-significant crude and adjusted ORs, although PCB-101 was significantly associated with PD risk (adjusted OR=2,74, 95% CI=1.27-5.92). Stratification by sex did not reveal any tendencies that differed between women and men.

Conclusions
Overall, these findings suggest that lifetime exposure to contaminants from traditional food, i.e. MeHg and POPs, contributed to the increased incidence of PD in the Faroe Islands. However, the data do not allow separation of a MeHg risk, or identification of possible joint effects of MeHg and POPs.

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WP I:6 Effects of manganese on the brain

Summary description of WP objectives
The objectives were: (i) To assess whether the population resident in Valcamonica, an area of historical operation of three ferroalloy plants (about one century until 2001), is still exposed to higher levels of metals released by ferroalloy, such as manganese (Mn), iron (Fe), lead (Pb), chromium (Cr), copper (Cu), zinc (Zn), compared to resident population in the reference area of Garda Lake, by measuring biomarkers and environmental exposure matrices; (ii) To verify exposure gradient of metals in relation to the previous point sources; (iii) To assess neuro-functional and neurobehavioral differences among the residents in the two areas as a result of different exposure levels to metals; (iv) To assess dose-response between metal exposure and health outcomes and to assess a geo-referenced gradient of health outcomes as a function of the point sources; (v) To verify the role played by genetic predisposition that may influence the health outcomes by interaction with exposure.

To implement the methodology for exposure and effects assessment, including genetic markers, a pilot study on active ferroalloy male workers and age-matched controls was also included.

Contractors involved
Bresc (WP leader Roberto Lucchini), LU, KI, UUC.

Degree to which the objectives were reached
The pilot study on workers allowed testing the validity of exposure and effects indicators and showed dose-effect responses between Mn exposure and impairment of motor/sway functions. A

possible protective cut-off of 100 µg/m^3 of inhaled total Mn was identified at this regard (Lucchini et al. 2008).

The five main objectives were fully achieved and this project has considerably increased the knowledge on neurotoxic effects of Mn from early life to the old age:

- Aim (i). After active emission ceased in year 2000, exposure levels to metals were still higher in Valcamonica, based on deposited dust (Zacco et al. 2009), soil, and air (Borgese et al. 2011) concentrations of Mn. Mn in blood was not related to inhalation exposure but to oral intake, both in children and elderly subjects. Preliminary results on Mn levels in deciduous teeth showed higher levels in enamel and coronal dentin corresponding to prenatal deposition, compared to the parts corresponding to postnatal deposition. The levels of Mn in both prenatal and postnatal parts were higher for children from Valcamonica, compared to the children from Garda Lake (Arora et al. 2011). Exposure analyses were extended to lettuce and chicorium, two types of salad commonly grown in home gardens in both areas of Valcamonica and Garda Lake. In the former area, lettuce showed higher Mn levels in the edible leaves, with a significant correlation to the soil levels. Further assessment of metal bio-accessibility, showed that the soil of Valcamonica was characterized by more soluble fraction for Mn, Pb, and other metals, compared to the soil of Garda Lake (Ferri et al., submitted).
- Aim (ii). According to soil levels, a south-north exposure gradient was observed in Valcamonica that may be related to the daily prevalent wind direction and the consequent transport of airborne particles.
- Aim (iii). Differences in motor coordination, odour identification, and body sway were observed for both children and elderly resident in Valcamonica, compared to the resident in the Garda Lake area. A decrease of children IQ was also noticed in the historically exposed area of Valcamonica.
- Aim (iv). Dose-effect-response showed decreased motor coordination, hand steadiness and odour identification as functions of Mn in surface soil among adolescents (Lucchini et al. 2011) and in airborne particles among elderly (Rentschler et al., submitted). Among adolescents, blood, hair and soil Mn were associated with tremor intensity (Lucchini et al., submitted), whereas blood-Pb was negatively associated with IQ and attention deficit/hyperactivity disorder (ADHD) scores (Zoni et al., submitted). The results of regression models were obtained after controlling for the influence of various covariates, including age, gender, socioeconomic status, and maternal education.
- Aim (v). Genetic predisposition was observed to influence the relationship between soil Mn and motor impairment among the elderly, as shown by the polymorphism of *ATP13A2* (*PARK9*) gene (Rentschler et al., submitted).

Methodologies and approaches employed

Several novel methods and approaches have been used in this WP for both exposure assessment in environmental matrices and biomarkers. Soil measurements have been achieved with a portable Niton XRay Fluorescence (XRF) instrument equipped with global positioning system (GPS) localization for quick assessment of metal concentration in the soil of the territory and data transfer onto digital maps of the territory. Airborne particulate was sampled with portable 24 hours SKC pumps, able to maintain a high flow of 10 l/min that yielded a complete assessment of indoor and outdoor environment that averaged about 2 and 22 hours, respectively, in children and elderly. Sampled membranes were transferred into a novel Bruker Total Reflection x-ray fluorescence (TRXRF) instrument, with a new procedure (Borgese et al., submitted) that was patented for international use (DePero et al. 2009). Measurements obtained with XRF and TXRF methodology were inter-calibrated with an inductively coupled plasma mass spectrometry (ICP-MS) method, showing good correlation. The soil bio-accessibility was assessed using a modified procedure of the BCR (Community Bureau of Reference) method issued by the EU Commission, and now known as SMTP (Standards, Measurement and Testing Programme). The extraction approach was not aimed to characterize environmental speciation *per se*, but to derive more predictive measure of metals in soil that better associate with biomarkers and health endpoints. Hair analysis was conducted after careful rinsing and sonication based on a new method that was able to minimize external contamination (Smith et al. 2011). Teeth analyses represented a totally innovative method, aimed to measure ^{55}Mn and the other metals in the prenatal and neonatal parts of deciduous teeth. The concentrations and spatial distribution of Mn in pre- and postnatal parts of enamel and coronal dentin were determined by laser ablation-ICP-MS, using a state-of-the-art ICP-MS and a laser ablation system. The analysis of dietary intake of Mn was particularly detailed through a specific food-frequency questionnaire, that was implemented with portions estimate, validated with weighting of individual food items. Motor, cognitive and sensory functions were assessed with a comprehensive state of the art testing battery (Zoni et al. 2007).

Achievements in relation to the state-of-the-art

This is the first epidemiological study on the effects of mixed elements, including Mn as main component, on neurological functions, where both inhalatory and dietary absorption are considered. Realized in a territory of Nothern Italy, where lifetime exposure to Mn has been related to the increased frequency of parkinsonian disturbances (Lucchini et al. 2007), this study has shown preclinical deficit in tremor, motor coordination and odour identification in workers, as well as in adolescents and elderly resident in the exposed area. Parkinsonian patients resident in the exposed area showed also higher levels of serum-Cu, and Mn in blood and urine, together with higher serum levels of enzymes indicating liver toxicity, as compared to age- and sex-matched healthy controls (Squitti et al. 2009). These health endpoints are suggestive as early signs of possible long-term neurodegenerative effects and were associated with Mn concentration in soil, as an indicator of historical/cumulative exposure. Genetic susceptibility was observed regarding the polymorphism of the gene *PARK9*, which is both related to Parkinson´s Disease and Mn toxicity. A possible cut-off protective level of 1,000 ppm was identified for total Mn in soil. Bio-accessibility evaluation showed higher metal solubility in the soil of the exposed area and higher transfer to edible plants like lettuce. For the exposed workers, a protective airborne level of 100 $\mu g/m^3$ of inhaled total Mn that was identified, based on the impairment of the same motor functions, as associated with cumulative exposure indices. Preliminary metal analysis in deciduous teeth was able to show higher pre- and postnatal levels among the resident in the exposed area. This result underlines the importance of early life vs. current exposure in the determination of neuro-developmental toxicity. The results of pregnant women showed higher Mn levels in cord blood compared to maternal blood; both were slightly higher in the exposed area. Being an essential element, Mn is needed by the developing brain but excessive absorption may induce neurotoxicity. Impaired cognitive abilities, as reflected by the IQ were associated with blood-Pb levels lower than 30 µg/L. The analysis of dose-response could not identify a protective cut-off, although a Benchmark Dose analysis for a loss of 1 IQ-point yielded a value of 20 µg/L, with a lower 95% confidence limit (BMDL) of 12 µg/L.

Results were presented at two conferences:

1. 10th International Symposium on Neurobehavioral Methods and Effects in Environmental and Occupational Health, San Josè Costarica, June 11-13, 2008: Lucchini R, Albini E, Zimmerman N, Zoni S, Marchetti S, Nan E, El Dalatony M, Borghesi S. Occupational exposure to manganese in ferroalloy industry: neurobehavioral effects in a workers' cohort. In: Abstracts of the p.186. Abstract.

2. ISEE Conference, Barcelana, Spain, September 13-16, 2011: Arora M, Bradman A, Lucchini R, Austin C, Vedar M, Holland N, Eskenazi B, Smith DR Deciduous tooth dentine as a biomarker of prenatal manganese exposure. Abstract.

Conclusions

The overall results offer novel information regarding the neurotoxicity of Mn across different age groups and in relation to inhalation exposure and dietary intake. Soil-to-plant transfer of Mn was identified as of possible concern for human health when exceeding the background levels.

The information gathered is, *inter alia,* useful for scientific advances and in view of the use of methyl-cyclopentadienyl manganese tricarbonyl (MMT), an organic derivative of Mn, as metal additive in fuel. The European Commission has restricted the use of MMT based on concern about possible health effects, and new scientific data are necessary for "an assessment of the risks for health and the environment from the use of metallic additives in fuel" (DIRECTIVE 2009/30/EC).

Publications

Borgese L, Zacco A, Pal S, Bontempi E, Lucchini RG, Zimmerman N, Depero LE. A New Non-Destructive Method For Chemical Analysis Of Particulate Matter Filters: The Case Of Manganese Air Pollution In Vallecamonica (ITALY). TALANTA, 2011 Mar 15;84(1):192-8.

Borgese L, Salmistraro M, Gianoncelli A, Zacco A, Bontempi E, Lucchini RG, Zimmerman NJ, Pisani L, Siviero G, Depero LE. Airbone Particulate Matter (PM) filter analysis and modeling by Total reflection X-Ray Fluorescence (TXRF) and X-Ray Standing Wave (XSW). Submitted

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WP I:7 Gene-environment interaction for effects of lead in children

Summary description of WP objectives

Children are very susceptible to the toxic effects of lead exposure, which may result in neurodevelopmental shortcomings. The susceptibility is varies inter-individually. Recent results indicate that the vulnerability may be modified by genetic polymorphism of the δ-aminolevulinic acid dehydratase (*ALAD*), and vitamine D receptor (*VDR*) genes. Hence, accumulation of lead in blood and other tissues may be modified, as well as lead toxicity.

Therefore, main questions were: Do *ALAD* and *VDR* genotypes modify lead toxicokinetics (blood-lead) and toxicodynamics (effects on cognitive function, hearing, posture and haem synthesis) in children?

Contractors involved

Sosno (WP leader Krystyna Pawlas), LU, KI.

Degree to which the objectives were reached

The objectives have been reached. One manuscript has been accepted and is pending revision.

Methodologies and approaches employed

In order to obtain an optimally wide exposure range to assess gene-environment interaction, study subjects have been recruited from two groups of children, the so called “old” and “new” cohorts.

The *“old” ’cohort* consists of two groups of subjects, who as children lived in Upper Silesia, in the vicinity of industrial lead emitters. One group had been examined for cognitive function, hearing and posture in 1994-1995 (155 subjects: 80 boys, 75 girls, aged 4-13 at the time of examination) and one in 2000-2003 (325 subjects: 183 boys and 144 girls, aged 3-11 at the time of examination).

The subjects in the "old” cohort were contacted again. Blood samples for genotyping were taken in 321 children (including 52 blood samples from a biobank established at the previous examinations).

To the *"new" cohort*, 301 children have now been recruited among 7-year old pupils of primary schools in the vicinity of industrial lead emitters in Upper Silesia.

The "new" cohort was examined according to the following protocol: (A) Questionnaire on background factors. (B) Biochemical laboratory tests [blood-lead level (B-Pb), genotypes for *ALAD* (the two single nucleotide polymorphisms (SNPs) rs 1139488 (*Rsa*I) and rs1800435 (*Msp*I G177C) and *VDR* (three SNP: rs1544410 (*Bsm*I), rs 10735810, *Fok*I and rs731236 (*Taq*I)], blood haemoglobin and zinc protoporfyrin (B-ZPP) levels, blood ALAD activity, serum cystatine, urinary creatinine, albumine, retinol-binding protein (RBP), total antioxidant status, 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxy), and TBARS. (C) A neurological test battery (pure-tone audiometry, impendance audiometry, transient acoustic otoemission, posturography, brainstem audiometric evoked potentials (BAEP), EEG spectrum and IQ measurement [Wechsler intelligence scale (Terman-Meril had been used in the "old" cohort, and was not used)].

Achievements in relation to the state-of-the-art

B-Pb differed between the two cohorts. In the "old" cohort (1996-2001), the median was 55 (range 23-470) µg/L, in the "new" one (2007-2010) 37 (9-269) µg/L. The median B-Pb was 42 µg/L in the merged cohort. B-Pb was significantly associated with the family's socio-economic status (both parents' education and income).

Genotype frequency: The detected frequency of genotypes was compared with the expected frequency, according to Hardy-Weinberg law, using χ^2. In the two *ALAD* genotypes and the three *VDR* genotypes, the allele frequencies did not differ in the "old" and "new" cohorts. The frequencies were similar to other European populations, but lower than in Asian populations (WP III:2).

B-Pb vs. genotype: In the merged cohort, the geometric mean B-Pb was 38.8 µg/L (range 9-470) µg/L. There were no significant differences in B-Pb for neither of the two *ALAD* genotypes. However, B-Pb was non-significantly higher in the *VDR Taq*I tt genotype (p=0.06) and decreased with increasing number of f alleles of *VDR Fok*I (p=0.08; Pawlas et al., manuscript).

Cognitive function vs. B-Pb and genotype: B-Pb was non-significantly negatively associated with IQ (r_S=-0.11; p=0.14). The *ALAD* genotype modified the relationship between IQ and B-Pb: *Rsa*I T carriers had a steeper slope, compared to CC homozygotes (p for interaction <0.001, adjusted for age and mother's education; Pawlas et al., submitted a). For *VDR Bsm*I, B carriers had a steeper slope than the bb homozygotes (p=0.023), while for *ALAD Msp*I, *VDR Fok*I and *VDR Taq*I there were no significant modifications.

Hearing vs. B-Pb and genotype: There were statistically significant associations between hearing thresholds (right ear, mean of 0.5-8 Hz), BAEP (V wave latency) and otoemission (TPC3K), on the one hand, and B-Pb, on the other (adjusted for sex, age, mother´s education, and mumps; Pawlas et al., manuscript a). Both *ALAD* and *VDR* genotypes modified the lead-induced effects on average hearing thresholds and BAEP. Hence, for *ALAD*, the variant genotypes (*ALAD*2 and *ALAD Rsa*I CC) suffered less effect than the others, even if the interactions were not statistically significant. However, for *VDR*, there were significant interactions: Two of the variant genotypes (*VDR Bsm*I B and *VDR Taq*I t) showed greater impacts on all effect parameters, while a third one (*VDR Fok*I f) was less susceptible.

Posture vs. B-Pb and genotype: Body sway measured by posturographic examination was associated with B-Pb (sway area with closed eyes: r_S=0.24, P<0.001; sway velocity: r_S=0.21, p<0.001; Pawlas et al., manuscript b). Significant effects (adjusted for the potential confounders height and mother´s education) were present already at B-Pb ≤50 µg/L, and then even stronger than at higher levels. The ff carriers in *VDR Fok*I polymorphism were more susceptible to toxic effect of lead on the balance system, while the other *VDR* or *ALAD* genotypes did not significantly modify the B-Pb effect.

B-Pb vs. EEG: B-Pb significantly correlated with increasing abnormality in EEGs and asymmetric distribution of EEGs. Further analyses, including modification by genes, are ongoing.

B-Pb vs. biochemical parameters: B-Pb was adversely correlated with ALAD activity and urinary total antioxidant status. Other examined biochemical parameters, like kidney function and heam synthesis onse, were not statistically significantly associated with B-Pb. Further analyses, including modification by genes, are ongoing.

Conclusions

1. The present lead exposure (B-Pb) in the lead-contaminated area was much lower than a decade ago; this is in accordance with studies in other areas. This is in accordande with results in WP III:I.
2. Effects on hearing functions in the route cochlea to brain stem were impaired by low-level lead exposure. Polymorphisms in the *ALAD* and *VDR* genes significantly modify the effects.
3. Effects on the central nervous system, as reflected by disturbancies of posture and cognitive function (IQ), were present already at very low lead exposure, in the case of posture even at B-Pb ≤50 µg/L; the effect at low levels was greater than at higher.
4. The frequencies of *ALAD Msp*I and *VDR* genotypes were comparable to earlier reports in European populations. This study gave frequency data on one additional *ALAD* genotype (*Rsa*I) , which has earlier been studied only occasionally.
5. *ALAD* and *VDR* genotypes significantly modified the relationships between B-Pb and toxic effects (IQ and posture).
5. Such gene-environment interactions imply that the meaning of B-Pb as an index of lead exposure and risk differs between individuals with varying genetic traits, and between populations with varying gene frequencies. This should be taken into consideration in risk assessment.
6. Children in the vicinity of lead emitters should be included in biomonotoring and preventive programs, with education on environmental safety behavior.

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WP I:8 Gene-environment interaction of elemental mercury

Summary description of WP objectives
The present WP aimed to seek for the presence of different gene-environment interactions in the metabolism (toxicokinetics) and neurological effects (toxicodynamics) of mercury (Hg) at exposure to elemental Hg (Hg^0).

Contractors involved
IFA (WP leader Raúl Harari), LU, IHEP

Degree to which the objectives were reached
All the objectives have been achieved in Study I (see below). In Study 2, the last steps data processing and publication are under way.

Methodologies and approaches employed
Ecuador (Study 1)
Hg^0 is used in the purification of gold by amalgamation in mining activities in many part of the world. This causes inhalation exposure to Hg^0 vapor at burning of gold amalgam to get rid of the Hg^0.

Ecuadorian gold buyers (N=37), gold miners (N=200) and referents (N=72) were studied. A detailed questionnaire on exposure and background information was used. Hg levels in whole blood (B-Hg), plasma (P-Hg) and urine (U-Hg) were measured (Partner LU). Levels of malone dialdehyde in serum (S-MDA) and 8-oxo-7, 8-dihydro-2'-deoxyguanosine in urine (U-8oxo) were determined (Partner IHEP) as markers of oxidative stress.

Tremor, postural sway, diadochokinesis and reaction time were assessed by a computerized equipment (same as in WP I:5).

Genotyping was made for a series of genes involved in the conjugation of Hg to glutathione (GSH), which is a step in its excretion through bile and urine.

Indonesia, Tanzania, Zimbabwe, Philippines (Study 2)
A total of 1,017 individual have been studied - gold miners, non-miners living in gold-mining communities (environmentally exposed) and in areas without gold mining (controls).

A detailed questionnaire on exposure and background information was used. Hg has been analysed in blood, hair and urine. The subjects have been examined for symptoms and signs of Hg^0 toxicity. Genotyping has been made for a long series of genes potentially involved in the metabolism of Hg - GSH-related ones, as well as several transporters.

Achievements in relation to the state-of-the-art
Ecuador (Study 1)
In gold miners, gold buyers and controls, the median U-Hgs were 3.3 (range 0.23-173), 37 (3.2-417), and 1.6 (0.2-13) µg/g creatinine, respectively (Harari et al., in press). The corresponding values for B-Hgs were 5.2 (0.7-100), 30 (4.4-89), and 5.0 (2.0-13) µg/L, and in P-Hg 1.9 (0.3-150), 21 (2.1-110), and 1.4 (0.5-6.8) µg/L. The concentrations in the buyers were statistically significantly higher than in the other groups; the miners differed from the referents in U-Hg and P-Hg.

There were no statistically significant associations between B-Hg, U-Hg and P-Hg, on the one hand, and S-MDA or U-8-oxo, on the other (Li et al., to be published).

There were few associations between Hg exposure (occupational, fish intake, amalgam fillings) and biomarkers of Hg (B-Hg, U-Hg, P-Hg), on the one hand, and results of computerized neuromotor examinations (tremor, hand coordination, reaction time, postural sway), on the other. However, B-Hg and U-Hg were associated with increases in the centre frequency of the tremor, as well as decreased reaction time and increased postural sway velocity.

Retention of Hg, as reflected by B-Hg, was modified by the gene for an enzyme in the glutathione synthesis (glutamyl-cystein ligase *GCLM*). The burning of gold amalgam among miners was intermittent. U-Hg decreased in the burning-free period. The elimination rate differed between individuals with polymorphism in the gene *GCLM* (half-times 77 *vs.* 32 days), while other polymorphisms in glutathione-related genes (*GCLC, GSTA1, GSTM1*, *GST-P1*) did not significantly modify the retention or elimination rate of Hg. Such modification is in accordance with findings in other parts of PHIME on the toxicokinteics of methylmercury (see below WP II:1.2).

There were no statistically significant modifications by the genotypes of the relationship between exposure (occupational, fish intake, amalgam fillings) or biomarkers of Hg (B-Hg, U-Hg), on the one hand, and the markers of toxic effects, S-MDA, U-8-oxo and neurotoxicity, on the other.

Indonesia, Tanzania, Zimbabwe, Philippines (Study 2)
The gold miners had a higher exposure than subjects living in gold ning communities, who - in turn - had higher exposure than the controls. Among the gold miners, those engaged in burning of gold amalgam were higher than the others. The Hg exposure was associated with toxic effects. This information has already been published by our collaborating parties.

The frequencies of genotypes in genes coding for Hg-transporters varied between the countries. However, we identified several genes that modified the levels of Hg biomarkers in several study groups from the different countries. Hence, effects were seen for variants of genes from the multidrug resistance (*MDR2*) and anion transporters families (Engström et al., manuscript). This information was presented at the 10th Conference on Mrcury as a Global Pollutant in Halifax, August 2011 (Engström et al. 2011). The potential modifications by the genes of the relationships between the biomarkers of Hg exposure/retention and toxic effects are presently analysed.

Conclusions

Hg exposure is a concern in gold miners in many parts of the world, and in the population living in gold-mining communities, because of environmental exposure. Among gold miners, the bruning of gold amalgam is the major source of exposure, and the body burden of Hg will vary over time according to such activities. Gold buyers, who burn daily, have continuous,high exposure.

The metabolism (toxicokinetics) of Hg is modified by genes involved in the conjugation of Hg to GSH (synthetases and transferases) and at least a couple of transporters. This means that subjects with similar exposure will obtain varying bod burdens of Hg,depending on their genetic predisposition, i.e. there is probably individual variation in vulnerability. Also, the meaning of biomarkers of Hg exposure/retention will vary between populations in different geographical areas.

In the Ecuadorian gold mines, there were few toxic effects, and no significant interaction between biomarkers of exposure/risk and markers of toxicity. Howeer, firm conclusions of whether genetic predisposition will affect the relationship between exposure/biomarker levels and neurotoxic effects must await the final statistical processing of data from Study 2

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Section 1.2
Pillar II Cardio- and cerebrovascular diseases, kidney and bone effects (Pillar coordinator Alfred Bernard)

Sub-Pillar II:1 Cardio- and cerebrovascular diseases (Sub-Pillar coordinator Karin Broberg Palmgren)

Mercury (Hg) is toxic to the cardiovascular system, an effect that could start already from fetal exposure to Hg. There are large differences between Sweden and Finland for association between Hg and myocardial infarction (MI). This may be due to differences of modifying factors, such as intake of fish fatty acids or genetic background. This Sub-Pillar has been broken down into three WPs: (1) Effects on prenatal and postnatal exposure to Hg on function of the cardiovascular system in a group of septuagenarians from the Faroe Islands, (2) Hg and risk of myocardial infarction (MI) or stroke, and effect modification of fish fatty acids, in Northern Sweden, and (3) Genetic factors influencing the effect of Hg on MI in Eastern Finland and Northern Sweden.

In summary, the results of Sub-Pillar II:1 indicate that high exposure to methylmercury through fish is a risk factor for MI, but the risk is modified by the simultaneous intake of PUFAs, which counteracts the methylmercury effect. Hence, the net risk is dependent upon the relative levels of methylmercury and PUFAs in the fish, and the amounts of fish consumed. Thus, the risk patterns vary with fish species, and between individuals and populations consuming varying amounts of fish, possibly also by the genetic traits of the population. Hence, the preventive actions (limits and advice) should vary in different countries.

WP II:1:1 Cardiovascular effects of methylmercury in the Faroe Islands

Summary description of WP objectives
Information on estimated prenatal and postnatal exposure to mercury (Hg) will be related to outcomes reflecting the function of the cardiovascular system in a group of septuagenarians.

Contractors involved
SDU (WP leader Philippe Grandjean), FSH

Degree to which the objectives were reached
Two third of septuagenarians invited participated in a thorough physical examinations and answered questions regarding their health and lifestyle history.

Methodologies and approaches employed
All the Faroese citizens, age 70 to 74 years, were invited by letter and eventually by telephone calls. Approximately two thirds agreed to travel to the clinic in the capital for hair, blood and toe-nail sampling, answering questionnaire and physical examination. The high participation rate among this group of elderly can partly be explained by the opportunity for a health check.

The prenatal exposure [to methylmercury (MeHg) and persistent organochlorine pollutants (POPs)] has been estimated by using whale-meat availability data from the area, where the mother of every single participant was living during the pregnancy. Lifetime exposure has been described by questionnaires. Present Hg exposure was measured in blood, hair and toenails. Furthermore, POPs are measured in serum. Fasting glucoses and insulin, together with vitamin D have been determined. Health history and clinical examination, including anthropometric data, blood pressure, ECG, heart rate variability, intima-media-thickness of common carotids have been obtained from 713 persons, age 70 to 74 years (according to the project protocol we initially only expected 500).

The statistical analyses of the relation between prenatal and postnatal Hg exposure and cardiovascular outcomes, as blood pressure, ECG abnormalities and intimae-media-thickness is ongoing.

Achievements in relation to the state-of-the-art
The achievements have been as planned.

Data have been presented at a conference:
Weihe P, Choi AL, Budtz-Jørgensen E, Salonen JT, Tuomainen TP, Murata K, Nielsen HP, Skaalum Petersen M, Grandjean P Methylmercury exposure and adverse cardiovascular effects in an elderly Faroese population. Oral presentation 10th International Conference on Mercury as a Global Pollutant (ICMGP) in Halifax, Nova Scotia, July 24. -29. 2011.

Conclusions
The results suggest adverse Hg effects on BP and IMT, although some of the associations were only close to significance. Adjustment with PCB, source of exposure from the consumption of whale blubber, did not substantially change the effects. The concentrations for this group of elderly people were much lower (less than half), compared with those of middle-aged Faroese men in a recent study. Separate analyses by gender found that the significant results were among men and not women and men had higher exposure levels than women, and also higher IMT measures.

Publications

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WP II:1:2 Mercury exposure as a risk factor for acute myocardial infarction and stroke – the Northern Sweden Study

Summary description of WP objectives

1. Do cases of myocardial infarction (MI) or stroke have increased blood levels of mercury (Hg)?
2. Do blood levels of Se or “fish fatty acids”, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), modify this relationship?

3. Are the risks modified by polymorphisms in enzymes involved in GSH synthesis or conjugation, or enzymes involved in the protection against oxidative stress?
4. Do genetic polymorphisms in pro- and antioxidative and detoxification genes modify this relationship?
5. Are there associations between blood Hg and biomarkers of oxidative stress (serum levels of 2F-isoprostane and peroxidised lipids)? Are such associations modified by polymorphism in enzymes involved in the protection against oxidative stress?

Contractors involved
UmU (WP leader Jan-Håkan Jansson), LU, UKU.

Degree to which the objectives were reached
Objectives 1-4 have been dealt with within the WP. Regarding Objective 5, it early became obvious that the marker of oxidative stress did not work in the way expected; hence, it was deleted.

Methodologies and approaches employed
Case-referent studies on MI and stroke have been performed by use of a biobank (with attached database on background and health parameters) containing samples from about 100,000 health-screened subjects. Hg levels have been determined in erythrocytes (Ery-Hg) in biobanked samples from cases and matched controls. The long chain n-3 polyunsaturated fatty acids EPA and DHA have been analysed in blood plasma (P-PUFA). For the genetic analyses, two different methods have been used; medium throughput using the Taqman assay, and high throughput using the bead chip technology from Illumina. Advanced multivariate statistical analysis and genetic association analysis have been employed in the different parts of this WP.

Achievements in relation to the state-of-the-art
Ery-Hg was low in this population; median levels were 3.63 µg/L in the stroke study (Wennber et al 2007; Wennberg 2010) and 3.54 µg/L in the MI study (Wennberg 2010; Wennberg et al. 2011). There were associations between intake on fish, Ery-Hg and P-PUFAs. This is because fish is the major source of methylmercy (MeHg) and PUFAs.

The Northern Sweden stroke study
In a prospective case-control study on 369 cases of stroke and 738 matched controls, no association was found between Ery-Hg and risk of stroke. Men reporting fish consumption >3 times/week had an elevated risk of stroke as compared to those reporting fish <1times/month, an association not found in women (Wennberg et al. 2007).

The Northern Sweden MI study
In a prospective study on 431 MI cases [including 81 sudden cardiac deaths (SCD)] with 499 matched controls, Ery-Hg was associated with decreased risk of MI. No significant associations with MI risk were found for reported fish consumption, EPA+DHA in plasma phospholipids or selenium in erythrocytes (Ery-Se). High levels of Ery-Se were associated with risk of sudden cardiac death (SCD) (Wennberg et al. 2011).

In a cooperation with Partner UKU, male MI cases from northern Sweden (Ery-Hg were transformed to hair-Hg and P-PUFA to serum-PUFA [S-PUFA]) were pooled with male MI cases from eastern Finland, with higher exposure to Hg. Preliminary data suggests that the fatty acids EPA and DHA modify the relationship between hair-Hg and MI risk (Wennberg et al., submitted).

Genetic studies
Individuals with certain genotypes for glutathione-related genes may tolerate higher exposures to Hg, due to faster metabolism/elimination and/or better glutathione-associated antioxidative capacity. Genotyping for GSH-related genes was done for 458 cases of first-ever MI and 569 matched controls. No significant genetic modifying effects were seen for the association between EPA+DHA in plasma phospholipids or Ery-Hg and MI risk. Still, our results indicate that the relatively rare *GCLM-588* TT genotype may have an impact, but a larger study is necessary for testing this hypothesis (Engström el al. 2011). Further genotyping, performed for more genetic markers associated with Hg metabolism, antioxidative capacity and MI have been performed for the same cases and controls, in collaboration with partner UKU. The genotyping has successfully been completed and statistical analysis is on-going.

Conclusions
In the low-exposed Northern Sweden population, no harmful associations were found between Ery-Hg and risk of stroke or MI. The finding on elevated stroke risk in men reporting high fish consumption will be followed up within a larger study. The protective agents in fish clearly overcome any possibly harmful effect of Hg-induced risk of MI at the exposure level in northern Sweden. The protective association between Ery-Hg and risk of MI is probably due to Ery-Hg being a good marker of fish consumption. The elevated SCD risk found at high levels of Ery-Se ought to be examined within a larger study. In the pooled study, with male MI cases from Finland and Sweden, preliminary data indicate that fish fatty acids, EPA and DHA, modify the effect of Hg on MI risk.

No significant genetic modifying effects of glutathione-related genes were seen for the association between Ery-Hg and MI risk. Analysis for further genetic markers with potential impact on MI risk is underway.

Publications
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Wennberg M, Strömberg U, Bergdahl I A, Jansson J-H, Kauhanen J, Norberg M, Salonen J T, Skerfving S, Tuomainen T-P, Vessby B, Virtanen J K. Myocardial infarction in relation to mercury and fatty acids from fish: A risk-benefit analysis based on pooled Finnish and Swedish data in males. Submitted.

WP II:1:3 Mercury exposure as a risk factor for acute myocardial infarction – the KIHD project

Summary description of WP objectives

The main objective is the analysis of single nucleotide polymorphism (SNP) * mercury (Hg) interactions in acute myocardial infarction (AMI) prediction in Eastern Finnish and Northern Swedish populations, utilizing high-throughput genotyping techniques. The results were to be compiled to scientific reports and publications. To achieve this, other key objectives were set as:

- Setting up a biobank for the Eastern Finnish population, selecting subjects according to existing phenotype information (Northern Swedish population samples were provided by Partner LU). For the genome-wide association study (GWAS), 200 cases and 200 controls were selected from Eastern Finnish population. For custom genotyping assay for Hg metabolism and AMI- associated polymorphisms, as well as for the Fine Mapping of the top hits (AMI; AMI * Hg; Hg) from a GWAS, 2,016 subjects were selected (including duplicates), half form the Northern Swedish, and half from the Eastern Finnish populations.
- Compilation of data on candidate SNPs and genes, associated with AMI and Hg metabolism (Hg-metabolism SNPs and genes were identified by LU).
- Producing novel and/or confirmed candidate SNPs for AMI/Hg metabolism, utilizing Whole Genome Scanning (WGS) of the Eastern Finnish population.
- Design of a combined custom genotyping panel (Illumina Infinium platform) for Hg-metabolism and AMI-associated polymorphisms and Fine Mapping of GWAS top hit polymorphisms, and screening these genotypes in the Eastern Finnish and Northern Swedish populations.

Contractors involved

UKU (WP leader Pekka-Tuomi Toumainen), UmU, JLB, LU.

Degree to which the objectives were reached

All objectives could be accomplished with 100 % success rate, in spite of the delays. Thus, the main key objective – a databank of genotype data of Eastern Finnish and Northern Swedish populations has been achieved. The data compiled in the Project will now make possible, in the course of next 1-2 years, to produce several new scientific key findings on this issue.

Methodologies and approaches employed

- Selection of subjects due to existing phenotype data, and establishment of a biobank of both archival and fresh samples.
- DNA-extraction method for archival samples, applying Qiagen DNA chemistry.
- Testing of the two leading genotyping platforms (Affymetrix and Illumina) for performance with archival samples, selection of Illumina due to excellent compatibility with the sample material of varying quality (DNA preparates of different ages, extracted with several different methods).
- Whole Genome Scanning (WGS), using Illumina Human 1M-Duo panel.
- Comparison of various genotype data administration and analysis platforms (SnpMax, Syllego, Progeny Lab, Exemplar Analytics, HelixTree and PLINK), of which PLINK was selected, to be used in combination with SPSS.

- Illumina GoldenGate OPA custom genotyping panel design for AMI and Hg metabolism associated SNPs, based on SNP and candidate gene information from literature, and a variety of genetic databases, utilizing, e.g., HapMap, HaploView, Ensembl and Illumina iCom tools.
- Statistical analysis of the WGS data with PLINK and SPSS, and selection of top candidate AMI/Hg metabolism SNPs for Fine Mapping.
- Compilation of AMI/Hg metabolism OPA panel with the Fine Mapping SNP panel derived from WGS data.
- Illumina Infinium iSelect custom genotyping panel design, based on SNP and candidate gene information from the literature, and a variety of genetic databases, utilizing, e.g., HapMap, HaploView, Ensembl and Illumina iCom tools.
- Genotyping of samples with Illumina Infinium iSelect platform with the custom SNP panel (6000 beads, providing 5532 SNP assays).
- Statistical analysis of the genomic data with PLINK and SPSS, including variety of statistical quality and validity checks of samples. Selected covariate-adjusted models of both WGS driven and literature based candidate genes and SNPs associated with AMI risks in both populations, and the pooled sample (main effects); same approach with Hg effects on AMI risk, and preliminary modelling of effect modification (interaction) of the set of promising candidate genes on the observed Hg effects in study populations.
-

Achievements in relation to the state-of-the-art

- Successful DNA extraction from archival samples.
- Successful selection of WGS and genotyping platforms, both compatible with a sample pool of varying quality.
- Successful WGS of Eastern Finnish samples.
- Successful genotyping of Eastern Finnish and Northern Swedish samples with a SNP panel for AMI and Hg metabolism.
- Successful Fine Mapping of Eastern Finnish and Northern Swedish samples.
- Successful statistical quality checks of the genotype data in both samples.
- Successful preliminary analysis of genotype data with respect to the risk of AMI, and the effect-modification (gene * Hg interactions).

The selected Illumina GWAS platform showed excellent performance with the heterogenous sample material, with an average call rate of 99.6% for 99% of the samples. Top hits of the GWAS included SNPs from genes involved in, e.g., energy and structural metabolism, as well as genes encoding various types of regulative proteins. As per number of top hit SNPs per chromosome, GWAS data indicated ‘hot spot’areas in chromosomes 1, 2, 3, 8 and 10. GWAS replication-candidate SNP analysis (i.e.’Fine Mapping’) of SNP panel selected by GWAS results was combined with the custom genotyping of AMI- and Hg-metabolism associated SNPs. In the preliminary analysis of the Infinium genotyping data, rather stringent filtration was used, leaving 4,052 acceptable SNP assays for 1,971 subjects (duplicates omitted).

In Fine Mapping, some interesting genomic areas for further study were identified. Strongest statistical associations, indicated by smallest p-value, were found in SNPs predicting body Hg in the Swedish cohort (SNPs in chromosomes 10 and 11 had p-values of 3×10^{-7} and 1×10^{-8}, respectively). For AMI, the statistical significances were not especially high, but loci for further evaluation were identified in chromosomes 8 (25% risk increase, both cohorts combined), chromosome 5 (30% risk reduction, Finns), and chromosome 4 (50% risk increase, Swedes). In AMI analyses with SNP * Hg interaction term, loci in chromosome 6 are promising (50% risk increase, combined cohorts).

Of the best 81 Fine Mapping SNP hits, 30 (37%) were replicants from the GWAS, and 51 (63%) were from the custom selected candidate genes SNP set.

Genomic analyses show that there are differences between the Swedish and Finnish populations in general, and in Hg-accumulation related SNPs. Also, the strongest AMI genes seem to differ between the two populations. The question whether the putative difference in cardiotoxicity of Hg between Finns and Swedes is explained by their genetic differences cannot be answered on the basis of the analyses carried out so far, but need yet further study. This work is continuing.

Conclusions

The new high throughput WGS and genotyping methods turned out to be very robust and compatible with sample material extracted with various different methods, and having remarkable variation in quality and storing time characteristics.

Genomic analyses show that there are differences between the Swedish and Finnish populations in general, and in Hg-accumulation related SNPs. Also, the strongest AMI genes seem to differ between the two populations. The question whether the putative difference in cardiotoxicity of Hg between Finns and Swedes is explained by their genetic differences cannot be answered on the basis of the analyses carried out so far, but need yet further study.

The PHIME co-operation around this study question has already produced a submitted manuscript that suggests the differences in the Hg and polyunsaturated fatty acids in the consumed fish between the two populations to be at least one of the important factors behind the observed difference (Wennberg et al., submitted).

Publication

Wennberg M, Strömberg U, Bergdahl I A, Jansson J-H, Kauhanen J, Norberg M, Salonen J T, Skerfving S, Tuomainen T-P, Vessby B, Virtanen J K. Myocardial infarction in relation to mercury and fatty acids from fish: A risk-benefit analysis based on pooled Finnish and Swedish data in males. Submitted.

Sub-Pillar II:2 Osteoporosis /fractures (Sub-Pillar coordinator Agneta Åkesson)

WP II:2:1 Exposure to cadmium and osteoporosis and
WP II.2:2 Exposure to cadmium and fractures

Because these WPs have extensive connections, they are here reported together.

Summary description of WP objectives

- Does cadmium (Cd) exposure through foods cause osteoporosis and fractures in populations living in areas without particular pollution?
- Possible mechanisms involved?
- Are there any interactions with nutrients or persistent organochlorine pollutants (POPs)?

- Does the genotype modify Cd retention or effects?
- Is the effect on bone reversible?

Contractors involved
KI (WP leader Agneta Åkesson), Fudan, UmU, Lund.

Degree to which the objectives were reached
All major objectives were reached.

Methodologies and approaches employed
We used a wide variety of study designs, ranging from cross-sectional studies over nested case-control studies to prospective cohorts and longitudinal (follow-up studies) (**Table 1**) to reach the objectives.

Exposure: Cd was analysed in whole blood, erythrocytes or urine. The dietary Cd intake was estimated from a food frequency questionnaire. POPs was assessed in serum (PCB-153 and *p,p´*-DDE).

*Outcomes***:** Bone mineral density of the forearm or at the total body including measurements of the hip, femoral neck and lumbar spine. Risk of osteoporosis, and risk of fractures and fracture incidence. Bone metabolic markers reflecting bone formation and bone resorption.

Additional analyses: Benchmark dose, to estimate the exposure level where the probability of effects on bone is low. Assessment of the active metabolite of vitamin D in serum, and serum retinol and urinary Cd and magnesium. Dietary intake of calcium and iron and dietary fiber. Polymorphisms in the methallothionein gene were assessed.

Table 1: Overview and major findings in the studies in **Sub-Pillar II:2**.

Cohort Area (Partner)	WP II:2	Study design and endpoints	Study population N	Major findings	Status
Southeast China (Fudan)	1	Longitudinal follow-up study 1998-2006. Forearm BMD recovery, Cd in blood and urine, bone metabolic markers, kidney markers.	458 men & women	No obvious recovery of low BMD after cessation of exposure. Prevalence of osteoporosis higher among women with prior tubular proteinuria.	2 papers published
Central Sweden I (KI)	1 (2)	Urinary Cd, dietary Cd intake, total body, hip & spine BMD, fractures (during 1997-2009). Assessment of benchmark dose for bone effects.	2,800 women	Cd in urine and diet associated with low BMD and increased risk of osteoporosis and fractures, the latter especially pronounced in never-smokers. Combined high dietary & urinary Cd associated with higher risk, indicating underestimation of risk in separate analysis of urinary or dietary Cd.	1 paper published, 1 submitted, 1 in preparation
Southern Sweden I (LU)	1	Erythrocyte Cd and serum POPs in relation to forearm BMD and bone metabolic markers.	908 women	Cd in erythrocytes inversely associated with BMD, which did not remain significant after adjustment for smoking. POPs not associated with BMD and there was no interaction with Cd.	1 paper published
Southern Sweden II (KI)	1	Urinary and blood Cd, serum-1,25(OH)$_2$ vitamin D, serum retinol, forearm BMD, bone metabolic markers. Assessment of benchmark dose for bone effects.	794 (vit D:116; serum retinol: 600)	Circulating active vitamin D not associated with Cd exposure. Serum retinol (vitamin A) seemed to counteract to some extent - the negative effect of Cd on bone.	3 papers published
Northern Sweden (UmU)	2	Cd in erythrocytes in a nested case-control of low trauma hip fractures.	111 fractures	Preliminary results: Increased risk of hip fractures that did not remain significant after smoking adjustment	Manuscript submitted
Central Sweden II Cohort (KI)	2	Prospective cohort. Dietary Cd intake and fracture incidence (2,200 any fracture and 374 hip fractures).	20,000 men	Increased risk of any fracture comparing highest tertile of dietary Cd with lowest. Increased risk of hip fractures among never-smoking men.	1 paper published

Cd=cadmium; BMD=bone mineral density; POPs=Persistent organochlorine pollutants (PCB-153 and *p,p´*-DDE).

Achievements in relation to the state-of-the-art

In a large cohort of 2,800 postmenopausal Swedish women with low environmental Cd exposure, we observed clear dose-dependent, statistically significant associations between Cd exposure and negative effects on bone (Engström et al. 2011 and Submitted). Multivariable-adjusted inverse associations were observed between both urinary and dietary Cd and BMD in the total body, femoral neck, total hip and lumbar spine. We also observed a statistically significant 2-3 fold increased risk of osteoporosis (T-score $\leq$2.5) per µg/g creatinine of urinary Cd, and per 10 µg/day of dietary Cd. Among never-smokers, a several-fold statistically significant increased risk of any first fracture, first osteoporotic fracture and first distal forearm fracture was observed for urinary Cd. A 30-50% statistically significantly increased risk of any first fracture were observed comparing high dietary Cd intake ($\geq$13 µg/day, median) with lower (<13 µg/day) among all women and never-smokers, respectively. Combined high dietary and high urinary Cd ($\geq$0.50 µg/g creatinine), as compared to low, three-fold statistically significantly increased risks of osteoporosis and fractures were observed among never-smokers. Thus, separate analysis of urinary or dietary Cd underestimated the risk.

During 10 years of follow-up, 2,183 cases of any fracture and 374 hip fractures were ascertained in a population-based prospective cohort of 20,000 Swedish men (Thomas et al 2011). Dietary Cd was associated with 19% increased risk of any fracture, when comparing the highest tertile with the lowest. In a risk-benefit analysis, we observed that men with high dietary Cd and low fruit and vegetable consumption, had a 41% higher rate of any fracture compared with contrasting tertiles. A 70% increased risk of hip fractures was observed among never-smokers.

In the nested case control study (biobank study), preliminary results indicate that association between erythrocyte Cd and hip fracture risk was partly removed after adjustment for smoking status (Rignell-Hydbom et al. 2009). Similarly, in another biobank study, comprising 900 Swedish postmenopausal women, Cd in erythrocytes was inversely associated with BMD, which did not remain significant after adjustment for smoking. Thus, erythrocyte Cd was not convincingly associated with causal adverse effects on bone. Furthermore, there was no support of any associations between the POPs (PCB-153 or *p,p´*-DDE) in serum and BMD and no interaction between these exposures and Cd.

In a mechanistic evaluation, we observed that decreased circulating levels of 1,25 dihydroxy vitamin D were unlikely to be the proposed link between Cd-induced effects on kidney and bone (Engström et al. 2009). In evaluation of interaction with nutrient, serum retinol (vitamin A) seemed to counteract some of the negative effect of Cd on bone (Engström et al., in press).

Benchmark dose assessment was applied to a urinary Cd and wrist BMD in a cohort of 794 women (age 53-64) from the South of Sweden (Åkesson et al. 2006), to estimate the exposure level where the probability of effects on bone is low (Suwasono et al. 2011).

In the Chinese cohort, with considerably higher past exposure to Cd, there was no obvious recovery of bone damage after cessation of exposure to Cd-polluted rice (Chen et al. 2009a and 2009b).

Conclusions

Altogether, the results provide important support that Cd exposure at the low levels found in the general population, with no particular exposure, is associated with negative effects on bone. This was indicated by decreased BMD, as well as increased risk of osteoporosis and fractures. The findings are of high public-health relevance, since the main dietary Cd exposure is *via* whole grains and vegetables, i.e. important foods, and that osteoporosis and osteoporotic fractures are prevalent in the population.

Thus, the PHIME project has substantially increased the database supporting an adverse effect of low environmental Cd exposure on bone. Unlike the studies on subclinical kidney effects – i.e. studies utilising tubular effect markers in terms of slight increase of low-molecular weight proteins, which has limited clinical relevance – the bone effects include several clearly health-relevant endpoints. Thus, they encompass clinical findings, the most severe being bone fractures. We suggest that the data on bone effects are more suitable for evaluation of health risks at low exposure levels than are those of kidney effects.

Irrespective of whether decreased BMD, increased risk of osteoporosis or fractures were assessed, these changes seem to occur at very low urinary Cd concentrations. Furthermore, we observed very clear increased risk of osteoporosis and fractures even among those who never smoked, demonstrating that the effect was not mediated via tobacco smoking. This was also strongly supported by the fact that the effects were associated with dietary Cd intake.

Publications

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Engström A, Michaëlsson K, Vahter M, Wolk A, Åkesson A. Associations between dietary cadmium exposure and bone mineral density and risk of osteoporosis and fractures among women. Submitted.

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Sub-Pillar II:3 Diabetes and kidney effects (Sub-Pillar coordinators Alfred Bernard/ Ingvar A Bergdahl)

Several elements of the periodic table are toxic to the kidneys. However, many aspects are not sufficiently well known, or open to discussion. Hence, six PHIME WPs studied markers of tubular and glomerular damage. Also, interaction between different elements and with another nephrotoxic agent (Ochratoxin A) was studied, as well as the reversibility of renal glomerular effects after a reduction of cadmium (Cd) exposure. Further, the ultimate clinical consequence of kidney toxicity - end-stage renal disease (uremia) was addressed.

WP II:3:1 Renal effects of mixed exposure to metals in teenagers

Summary description of WP objectives
Heay metals polluting the environment can cause renal effects (e.g. slight urinary excretion of low-molecular weight proteins) on vulnerable populations, but it is uncertain whether these metals still pose health risk at the very low exposure levels now prevailing in Europe. The objective was to assess the health significance of an association between proteins and cadmium (Cd) and lead (Pb) in the urine at low exposure.

Contractors involved
CAT (WP leader Alfred Bernard), LU, PPSE.

Degree to which the objectives were reached
The objectives have been achieved.

Methodologies and approaches employed
In a cross-sectional study of 736 adolescents aged 13.7 to 17.9 years, we have assessed the associations between the concentrations of Cd and lead (Pb) in blood or urine and the urinary concentrations of retinol-binding protein (RBP), β_2-microglobulin and albumin. Multiple regression analysis models were tested by adjusting urinary markers for urinary creatinine or specific gravity.

In order to confirm the findings, the study was repeated in 126 Moroccan teenagers.

Achivements in relation to state-of-the-art
The median metal concentrations in the Belgian teenagers were in blood (µg/l): Pb, 15.1; Cd, 0.18, and in urine (µg/g creatinine): Cd, 0.09; Pb, 0.82 and Hg, 0.55 (Chaumont et al., manuscript). The multivariate analyses revealed very significant associations in the urine ($p<0.001$) between RBP and Cd as well as between β_2-microglobulin and Pb, both in the creatinine and the specific gravity models. These associations were, however, completely abolished in subjects with increased urinary albumin, which may be explained by the competitive inhibition of low-molecular-weight protein reabsorption by albumin. Such associations were neither seen with the blood concentrations of Cd and Pb, nor with urinary Hg.

The Moroccan teenagers had about twice as high urinary Cd concentrations, as compared to the Belgian youngsters. This is in accordance with the findings in children and women from different European countries and Morocco (WP III:1). There were no statistically significant correlations between U-Cd and the renal markers albumin (**Figure 1**) or RBP (**Figure 2**). Still, it is of interest that there are non-significant associations at these low levels. This is in accordance with the findings in the Belgian teenagers, and lends some support to the conclusion, meaning that there may be a reverse causality, so that proteinuria may increase the excretion of Cd.

Conclusions

Associations between low-molecular-weight proteins and Cd or Pb in urine at very low environmental exposure levels should be interpreted with caution, given the unspecific nature of the tubular reabsorption of proteins. The close relationships between low-molecular-weight proteins and Cd or Pb in urine might simply reflect the inter-individual variations in the tubular reabsorption capacity of proteins.

Publication

Haddam N, Samira S, Dumont X, Taleb A, Lison D, Haufroid V, Bernard A. 2011. Confounders in the assessment of the renal effects associated with low-level urinary cadmium: an analysis in industrial workers. Environ Health.2011; 14:10:37.

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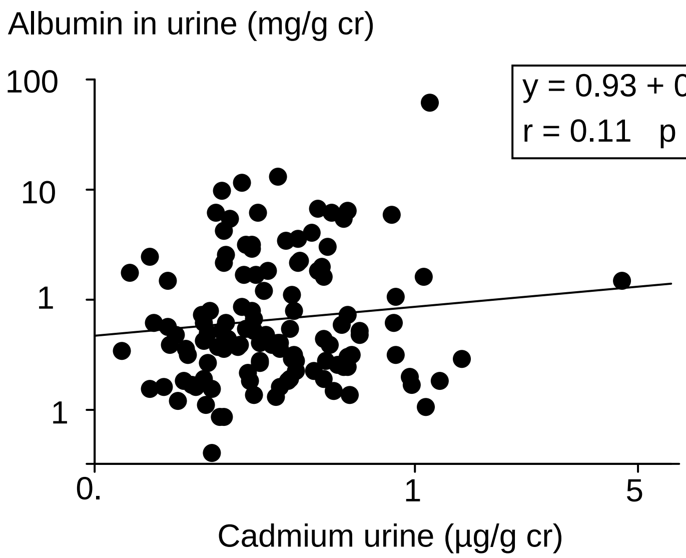

Figure 1. Relationship between albumin and cadmium concentrations in urine in 126 Moroccan teenagers.

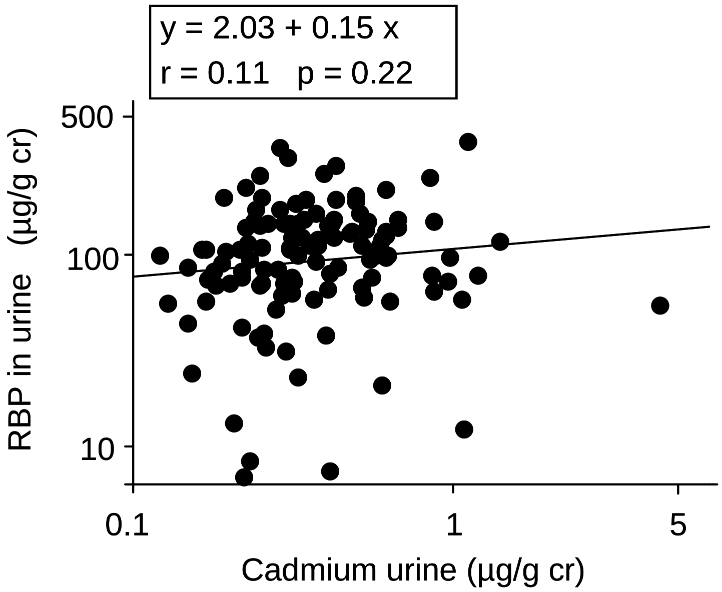

Figure 2. Relationship between retinol-binding protein and cadmium concentrations in urine in 126 Moroccan teenagers.

WP II:3:2 Kidney damage and diabetes from mixed exposure to metals

Summary description of WP objectives
The overall aim was to clarify to what extent simultaneous chronic environmental exposure to nephrotoxic elements, affect kidney function (identified by analyses of biomarkers of glomerular and tubular damage) in men, women and children/adolescents, and to investigate if malnutrition or occurrence of diabetes would further potentiate the nephrotoxic effects of the toxic elements.

Contractors involved
KI (WP leader Marika Berglund), ICDDR,B, CAT, LU.

Degree to which the objectives were reached
All the planned activities were performed.

Methodologies and approaches employed
The study is part of a population-based case-referent study concerning the risk for skin lesions in relation to arsenic (As) exposure via drinking water, carried out in Matlab, a rural area 53 km south-east of Dhaka, Bangladesh (Rahman et al. 2006). Only randomly selected referents were studied. The study was based on analyses of previously collected spot urine samples within the large epidemiological study "Arsenic in tube-well water and health consequences in Matlab, Bangladesh" (AsMat).

A database was constructed including children aged 8-12 years, adolescents aged 14-15 and adults aged 30-88. Information regarding smoking habits and tobacco use, socioeconomic status, body mass index (BMI), etc., was obtained from the comprehensive Health and Demographic Surveillance System (HDSS) in Matlab, run by the International Centre for Diarrhoeal Disease Research (ICDDR,B), Bangladesh.

Metal concentrations were analyzed in spot urine samples, previously collected, using inductively coupled plasma mass spectrometry (ICPMS; Agilent 7500ce, Agilent Technologies, Waldbronn, Germany) with a collision/reaction cell system, operating in hydrogen (Se), helium (As, Ca, Cd, Fe, Mg, Mn, Zn) or standard mode (Hg, Mo, Pb, Sb, U) (Kippler et al. 2007 and 2009). To compensate for variation in urine dilution, concentrations were adjusted to the mean specific gravity (1.012 g/mL). Specific gravity was measured by a refractometer (Uricon-Ne, ATAGO Co. Ltd, Tokyo, Japan). Urinary creatinine was determined using the Jaffe method (Hare 1950).

Plasma ferritin and zinc (Zn) were determined in a subsample (in about 30% of the population). Ferritin was measured in plasma using radioimmunoassay (Diagnostic Products, San Diego, CA, USA). Zn was assessed in plasma by atomic absorption spectrometry (Clegg et al. 1981).

Achievements in relation to the state-of-the-art
An unexpectedly high number of analytical results for kidney effect markers [albumin, retinol-binding protein(RBP)] in urine were below detection limits (about 50% of all samples; analyzed at Partner CAT). Furthermore, the urine creatinine concentrations (about 0.6 g/L in adults and 0.4 g/L in children), as well as the urine specific gravity (mean 1.012 g/mL), indicated that the urine samples were highly diluted. A thorough investigation, including reanalyses of a subsample of urines, was performed to elucidate possible reasons for the low urinary concentrations of creatinine and kidney effect markers.

The creatinine excretion in urine is proportional to both the muscle mass and the nutritional status, i.e. a low muscle mass is reflected by a lower creatinine excretion. Indeed, 30% of the adult population in our study had a BMI below 18.5 kg/m^2 (indicating undernutrition), and 50% of children and adolescents were classified as underweight according to growth charts using BMI (Kuczmarski et al. 2000). The creatinine levels were significantly associated with the body size [BMI, body surface area (BSA)] of both adults and children. The low creatinine concentrations are most probably reflecting the low lean body mass, and also a general chronic malnutrition.

It is possible that degradation of proteins/effect markers in urine had taken place to a certain extent. Even if the urine samples were kept in portable coolers immediately after collection and kept frozen until analysis, it took several years until the effect markers could be analyzed. In general, creatinine and the kidney markers analyzed are stable in urine at physiological pH. However, pH in urine was positively associated with the kidney effect marker concentrations in a subsample that was reanalyzed, and it seemed that degradation started already at pH 6. It is also possible, however not tested, that some adsorption of proteins to the test tube walls may have occurred which would give erroneously low results.

It was concluded that the kidney effect marker data could not be used, and that no final evaluation of kidney effects from mixed metals exposure could be performed.

We found very low urinary creatinine concentrations in men, women and children. Differences in lean body mass, dietary habits, and in particular status of nutrition, e.g., chronic undernutrition, will result in differences in urine creatinine, not only between adults and children but also between populations. These variables need to be considered when creatinine corrected urinary results are compared between studies. Furthermore, we found that creatinine in urine was significantly positively correlated with all the metals/elements analyzed in urine, except manganese (Mn), which was significantly negatively correlated with creatinine in urine in children, and not correlated in the other age groups. The results indicate that creatinine and metals are co-excreted in urine which needs to be considered when metals in urine are used as markers of exposure.

We also found quite a range in toxic metal and element concentrations in urine, and of which all analytical results were above detection limits (analyzed at KI). Thus, we decided to investigate gender and age differences in the exposure to the toxic elements As, Cd, Pb, Sb and U, as well as the essential elements Ca, Mg, Fe, Zn, Mn, Mo and Se, by evaluation of the element concentrations determined in urine. Little is known about the variation in exposure to toxic metals by age and gender and the interactions with nutrition and other potential modifying factors such as socioeconomic status (SES), BMI, and smoking habits. Indeed, we found marked differences in metal exposure and trace element intake (measured as urine concentrations) by gender, age, tobacco use, socioeconomic and nutritional status. A manuscript "Gender and age differences in mixed metal exposure and urinary excretion" by Berglund et al., will be submitted shortly.

Conclusions

It was concluded that the kidney effect marker data, of which about 50% of the results were below detection limits, could not be used for the evaluation of kidney effects from mixed metals exposure. We found very low creatinine concentrations and specific gravity in urine in men, women and children, indicating that the urine samples were highly diluted. The low creatinine concentrations are most probably reflecting the low lean body mass and also a general chronic malnutrition. We found marked differences in metal exposure and trace element intake (measured as urine concentrations) by gender, age, tobacco use, socioeconomic and nutritional status.

Publications

Kippler M, Ekstrom EC, Lonnerdal B, Goessler W, Åkesson A, El Arifeen S, et al. 2007. Influence of iron and zinc status on cadmium accumulation in Bangladeshi women. Toxicol Appl Pharmacol 2007;222:221-226.

Kippler M, Lonnerdal B, Goessler W, Ekström EC, Arifeen SE, Vahter M. 2009. Cadmium interacts with the transport of essential micronutrients in the mammary gland - a study in rural Bangladeshi women. Toxicology 2009;257:64-69.

Rahman M, Vahter M, Wahed MA, Sohel N, Yunus M, Streatfield PK, et al. 2006. Prevalence of arsenic exposure and skin lesions. A population based survey in Matlab, Bangladesh. J Epidemiol Community Health 2006;60:242-248.

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Clegg MS, Keen CL, Lönnerdal B, Hurley LS. Influence of ashing techniques on the analysis of trace elements in animal tissue. Biol Trace Elem Res 1981;3:107-115.

Hare RS. 1950. Endogenous creatinine in serum and urine. Proc Soc Exp Biol Med 1950;74:148-151.

Kuczmarski RJ, Ogden CL, Grummer-Strawn LM, Flegal KM, Guo SS, Wei R, et al. 2000. CDC growth charts: United States. Adv Data 2000;:1-27.

WP II:3:3 Exposure to cadmium, lead and mercury and end-stage renal disease

Summary description of WP objectives

- Is end-stage renal disease (ESRD; uraemia) associated with exposure to some toxic elements in the population?
- Are there gene-environment interactions?

Contractors involved

UmU (WP leader Ingvar A. Bergdahl), PPSE, LU, CAT.

Degree to which the objectives were reached

Associations can be seen, in preliminary analyses of collected data, between lead (Pb), possibly also cadmium (Cd), and the risk of attaining ESRD later in life. The first part of the objectives is fulfilled, but no studies of gene-environment interactions have been performed.

Methodologies and approaches employed

118 cases, who had later developed ESRD, and 378 individually matched (age, sex, and time of blood sampling) referents were identified among participants in two population-based cohorts (130,000 individuals), who had donated blood at a health examination. Metals in erythrocytes (Ery) were determined by inductively coupled plasma-mass spectrometry (ICP-MS; Cd and Pb) or cold vapour atomic fluorescence (CVAAS; Hg). The erythrocytes were sampled prior to ESRD diagnosis [mean time from baseline examination to ESRD diagnosis: 7.7 years (range: 1-16)].

Achievements in relation to the state-of-the-art

Preliminary analyses of this prospective population-based study indicates that exposure to Pb - and perhaps Cd - predict future risk of developing ESRD in the general population and that low-level concentration of the elements in blood predicts the risk of ESRD (Nilsson et al., submitted). Suggestions of this have previously been provided from cross-sectional studies, but this study is the very first study of prospective character.

The risk relation was only slightly changed when analysis was restricted to individuals with blood samples donated more than 10 years prior to development of ESRD. Therefore, the Pb exposure appears to precede the development of ESRD. However, it seems unlikely that Pb alone is the cause of this finding. An alternative explanation is that Pb may decrease the kidney's ability to resist various strains and challenges. In addition, Ery-Cd tended to be related to an increased risk of ESRD, but the major part of this association was explained by confounding by Pb exposure. Ery-Hg, in contrast, shows a negative association, with a reduction in the risk of ESRD.

Previous cross-sectional studies indicate that patients with chronic renal failure have increased retention of Cd and Pb (Sánches-Fructuoso et al., 1996; Lee et al. 2000; Muntner et al. 2007; Kazi et al. 2008; Chen et al. 2009), and that Pb is related to the progress (Lin et al. 2001). However, such findings may also be a result of reversed causality; the disease itself, or its treatment may affect metal retention. The study performed within PHIME is most probably the first prospective study of biomarkers of metals exposure in relation to ESRD. Its result are therefore of great importance for the assessment of risk for actual kidney disease in relation to metals.

Conclusions
The first prospective study of biomarkers of metals exposure in relation to ESRD has been performed. Preliminary data analyses indicate that exposure to Pb - and perhaps Cd - predicts future risk of developing ESRD in the general population, i.e. at low levels. This gives reasons to suspect that exposure to Pb - and maybe also Cd - contribute to the development of ESRD.

Publication
Nilsson J, Svensson M, Björ B, Elmståhl S, Hallmans G, Lundh T, Schön S, Skerfving S, Bergdahl IA. End-stage renal disease and low exposure to lead, cadmium and mercury – a populations-based, prospective nested case-referent study in Sweden. Submitted.

WP II:3:4 Reversibility of cadmium-induced kidney damage

Summary description of WP objectives
Long-term exposure to cadmium (Cd) causes renal dysfunction, but how these effects change upon cessation from, or reduction in, Cd-exposure is unknown: An issue of concern is whether today's exposure will affect the future life of the individuals, or if the effects are limited to the near time-period.

The aim of the study was to find out how the prevalence of impaired kidney function develops after cessation of exposure, and how that development relates to the initial exposure level and other characteristics, such as age and sex. We studied this for three different biomarkers of kidney function: urinary *N*-acetyl-β-D-glucosaminidase (U-NAG), β_2-microglobulin (U-B2M), and albumin (U-ALB). For these biomarkers we also investigated how predictive an impaired level is for future impairment in the same individual.

Contractors involved
Fudan (WP leader Taiyi Jin), UmU, LU.

Degree to which the objectives were reached
All planned activities have been performed. One manuscript has been published, and two submitted. Further data analyses will be made by UMU and Fudan.

Methodologies and approaches employed
A pilot study was carried out on 148 individuals (Wu et al. 2008). It was followed by a study of 475 residents (412 when restricting to those of age <80 years and systolic pressure <170 mmHg) examined both in 1998 and 2006. They lived in one of three areas with previous Cd pollution of varying degree: highly, moderately or non-polluted areas (**Table 1**).

At both occasions, blood (B-Cd) and urinary (U-Cd) levels were analysed, as well as U-NAG), U-B2M), and U-ALB. Genotyping was made for methallothionein genotype *MT1A*.

Achievements in relation to the state-of-the-art
The results of the pilot study suggested that the Cd-induced renal dysfunction might be reversible if U-Cd was low before exposure decreased, otherwise it might be irreversible or aggravated. However, due to the small number of individuals and the short follow-up time (3 years), firm conclusions could not be drawn.

The major study's results on biomarkers of Cd exposure and nephrotoxicity are presented in **Table 1** and Liang et al. (submitted). From B-Cd data it is evident that the exposure in the highly exposed area had decreased greatly from 1998 to 2006 and a decrease is also evident for the moderately polluted area.

U-ALB showed a dose-response relation to Cd in 1998, but had in 2006 returned to normal (**Figure 1c**). Other biomarkers of kidney function were also elevated in 1998 but only partly returned to normal upon decreased exposure (**Figures 1a-b**). Using age-adjusted cut-off values, partial recovery was observed for U-NAG among women, and suggested for U-B2M among young individuals (data not shown here, Liang et al., submitted; D II:3:4.6).

The risk of having impaired U-B2M in 2006 was increased in those with an impaired value in 1998 (odds ratio=OR: 24.8, 95% CI: 11.2-55.3). Corresponding ORs for U-ALB: 3.0 (1.2-7.5), and for U-NAG: 2.6 (1.6-4.4).

There was a modification by methallothionein genotype *MT1A* for kidney toxicity of Cd (Lei et al., submitted).

Conclusions

This study over eight years shows clearly that albuminuria caused by Cd exposure (at the observed exposure levels) is a reversible effect that recovers after cessation of exposure. For the markers of tubular effects, a tendency towards improvement, but not complete recovery, was observed for U-NAG, but only among men. Maybe there is also some improvement for U-B2M), but then only in relatively young individuals.

On the individual level, the study demonstrates that an increased U-NAG excretion has very little prognostic value for the result of a future sampling. In contrast, a rise in U-B2MG excretion is a strong predictor of impaired values also in the future.

There is a possibility of a gene-environment interaction for Cd nephrotoxicity, which may be important from a risk assessment point of view, and which should be further explored.

Publications

Liang Y, Lei L, Nilsson J, Li H, Nordberg M, Bernard A, Nordberg GF, Bergdahl IA, Jin T. Renal Function after Reduction in Cadmium Exposure: An Eight-Year Follow-Up of Previously Studied Residents in Cadmium-Polluted Areas. Submitted.

Lei L, Chang X, Tian L, Zhu G, Chen X, Rentschler G, Jin T, Broberg K. A polymorphism in metallothionein 1A (MT1A) is associated with cadmium-related kidney toxicity. Submitted.

Wu X, Liang Y, Jin T, Ye T, Kong Q, Wang Z, Lei L, Bergdahl IA, Nordberg GF. Renal effects evolution in a Chinese population after reduction of cadmium exposure in rice. Environ Res 2008;108:233-238.

Table 1. Levels of exposure and renal toxicity biomarkers in 1998 and 2006, by Cd-pollution area.

	Followed in 2006			Lost to follow up in 2006	
	1998	2006	*p**	1998	*p*#
Non-polluted area					
N (males) (%)	123 (34.1)			130 (43.1)	
Age (median, in 1998)	58.0			52.0	<0.05
B-Cd (μg/L)	1.35 (0.75, 2.13)	0.91 (0.59, 1.46)	<0.01	1.47 (0.95, 2.19)	0.340
U-Cd (μg/g Cr)	1.91 (1.19, 3.64)	2.40 (1.45, 4.10)	0.131	1.75 (1.24, 3.00)	0.262
U-NAG (U/g Cr)	1.97 (0.95, 4.48)	9.33 (6.01, 12.5)	<0.01	1.86 (1.04, 5.04)	0.804
U-B2M (mg/g Cr)	0.14 (0.07,0.26)	0.20 (0.10, 0.34)	<0.01	0.19 (0.09,0.34)	0.058
U-ALB (mg/g Cr)	3.09 (1.50, 6.40)	2.93 (1.31, 6.48)	0.676	3.03 (1.38, 5.75)	0.708
Moderately-polluted area					
N (males (%)	144 (33.3)			99 (33.3)	
Age (median, in 1998)	46.0			47.0	0.993
B-Cd (μg/L)	4.15 (2.53, 6.63)	1.82 (1.25, 2.74)	<0.01	3.04 (1.88, 5.00)	<0.01
U-Cd (μg/g Cr)	3.62 (2.54, 6.00)	3.81 (2.66, 6.14)	0.976	3.45 (2.46, 6.61)	0.996
U-NAG (U/g Cr)	3.84 (1.88, 11.2)	8.24 (6.01, 10.84)	<0.01	3.16 (1.53, 10.6)	0.337
U-B2M (mg/g Cr)	0.16 (0.10, 0.28)	0.28 (0.17, 0.42)	<0. 01	0.17 (0.10,0.29)	0.456
U-ALB (mg/g Cr)	4.56 (2.03, 10.15)	3.98 (1.40, 9.63)	0.544	4.02 (2.00, 8.60)	0.337
Highly-polluted area					
N (males (%)	208 (37.5)			86 (52.3)	
Age (median, in 1998)	47.0			55.5	<0.05
B-Cd (μg/L)	9.00 (5.00, 14.1)	3.36 (2.30, 5.15)	<0.01	9.20 (5.28, 15.1)	0.678
U-Cd (μg/g Cr)	11.4 (7.31, 17.9)	8.98 (5.85, 13.4)	<0.01	10.7 (6.2, 19.0)	0.531
U-NAG (U/g Cr)	7.47 (4.29, 14.1)	11.9 (7.32, 17.8)	<0.01	9.70 (4.31, 24.4)	0.123
U-B2M (mg/g Cr)	0.28 (0.14, 0.52)	0.44 (0.21, 0.80)	<0.01	0.49 (0.14,1.39)	<0.01
U-ALB (mg/g Cr)	5.31 (2.50, 11.8)	3.57 (1.43, 8.64)	<0.01	7.82 (3.85, 18.2)	0.029

These data were reported as geometric mean and IQR (interquartile range). Cr=creatinine.

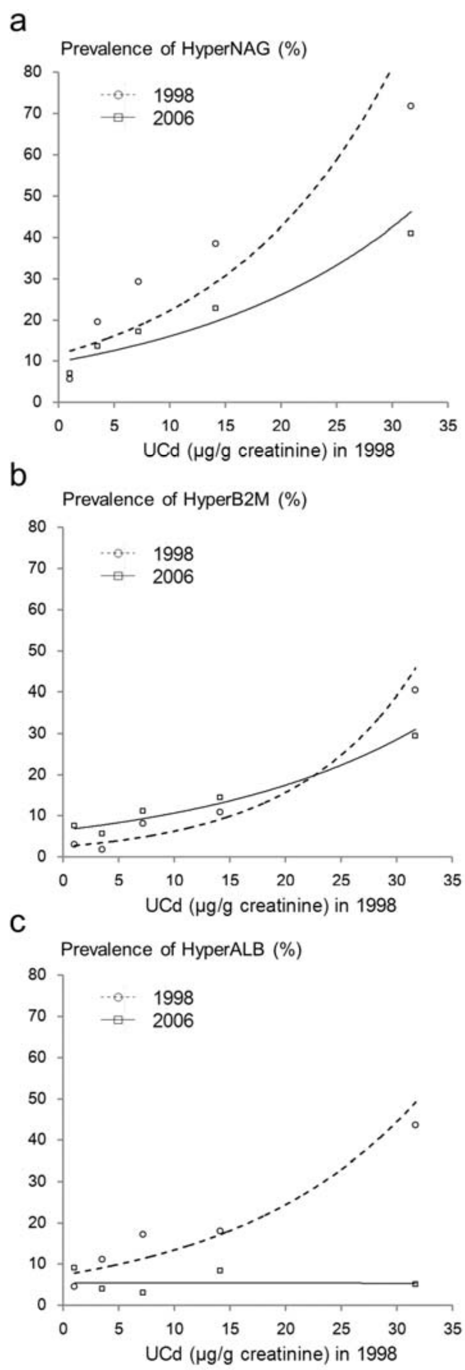

Figure 1. Prevalences of renal dysfunction (dashed line: 1998, solid line: 2006) against the U-Cd in 1998. HyperNAG (a), HyperB2M (b) and HyperALB (c) refer to the abnormally high levels of *N*-acetyl-β-D-glucosaminidase, β_2-microglobulin and albumin (above the cut-off values), respectively. The results imply that the population has recovered from Cd-induced effects on U-ALB but only a minor recovery is suggested for U-NAG and U-B2M.

WP II:3:5 Exposure to cadmium and diabetes

Summary description of WP objectives
Previous studies indicate higher Cd exposure among individuals with type 2 diabetes mellitus (DM), compared to referents, but no studies of prospective design have been presented.

So, the objective was: Is Cd exposures associated with risk of DM any type?

Contractors involved
UmU (WP leader Ingvar A. Bergdahl), LU, CAT.

Degree to which the objectives were reached
Data have been collected and preliminary analyzes performed.

Methodologies and approaches employed
Cd was determined in erythrocytes (Ery-Cd) stored in a biobank (and an attached databank on health and background information) containing 130,000 samples collected at earlier health screenings. Cases were 164 individuals, who acquired diabetes mellitus (DM) after sampling. These were compared to 298 matched (sex, age, time of sampling) referents without diabetes.

Achievements in relation to the state-of-the-art
Preliminary analyses of our data show mean Ery-Cd: 1.28 (SD: 1.80) µg/L among cases, and 1.04 (SD: 1.70) among referents. The risk of DM was not associated with Ery-Cd, but in separate analyses for men and women, a tendency appeared for men only: In an adjusted model, odds ratio=OR was 1.24 (95% CI: 0.76-2.00) per 1 µg/L increase of Ery-Cd. A manuscript is in preparation.

Conclusions
We can not prove an association between risk for DM and Ery-Cd. Neither can we negate the existence of an association. Men may constitute a sensitive group.

WP II:3.6 Interaction between nephrotoxic metals and Ochratoxin A

Summary description of WP objectives
In the Mediterranian area, there are often problems with high contamination of vegetable foods with the mycotoxin Ochratoxin A (OTA). Both OTA and heavy metals [cadmium (Cd), lead (Pb), mercury (Hg)] are nephrotoxic, causing proximal tubular damage.

The objective was to find out whether those agents interact with regard to kidney effects.

Contractors involved
PPSE (WP leader Baadia Lyoussi), CAT, UmU, LU.

Degree to which the objectives were reached
All objectives have been achieved. Final statistical processing is in progress.
In addition to the original aims, a survey of OTA levels in foods in Morocco has been performed.

Methodologies and approaches employed

- 50 women (age 50-60) from each from a rural, urban and industrial area in Morocco were examined.
- A questionnaire on health and backround information was administered.
- Blood and urinary samples were obtained.
- Analysis of Cd, Pb, and Hg in blood (B-Cd, B-Pb and B-Hg, respectively) and urinary (U-Cd, U-Pb and U-Hg, respectively) were made, after quality control analysis done in Brussels, has been performed in collaboration with CNESTEN, Rabat.
  Analysis of an OTA metabolite in serum (S-OTA) was performed in Toulouse by HPLC.
- Analysis of markers of kidney toxicity [serum creatinine and albumin, and urinary creatinine, albumin and retinol-binding protein=RBP)] was made by Partner PPSE in collaboration with Partner CAT.
- Food samples (cereals, bread and spices) were sampled in Fez–Boulemane Region, and analysed for OTA.

Achievements in relation to the state-of-the-art

Metal levels in blood are presented in **Figure 1**. B-Pbs were much higher in the industrial area than in the urban and rural ones ($p<0.001$; analysis of variance), in which the levels were still higher than in most European settings (see data below on women in seven European countries studied in WP III:1), probably because of remaining use of Pb in petrol. B-Cd were much higher in the urban area than in the rural and industrial ones, for which the levels were higher than in most European areas. The explanation is probably a higher intake of rice, which usually contains more Cd than other foods. The urban values may possibly be due to the presence of smokers; tobacco is a major source of Cd. B-Hg was lower in the rural area than in the urban and industrial ones. B-Hg was higher than usually seen in Europe, probably because of a high intake of fish, which is a major source of methylmercury.

S-OTA data are given in **Figure 1**. The levels were much higher in the industrial area than in the rural and urban ones ($p<0.001$).

The urinary albumin levels varies between the three areas ($P<0.001$; not shown). The other kidney toxicity markers did not differ between areas.

There were no statistically significant associations between levels of any of the three metals in blood or S-OTA, on the one hand, and the kidney toxicity markers, on the other. As examples, relationships between S-OTA and U-RBP are shown in **Figure 2.**

Data on OTA levels in Moroccan foods are presented in **Table 1**. The highest levels were found in bread (4.30 ± 1.2 ng/g; 1.52 -20.19 ng/g). This is higher than the maximum limit (3 ng/g) set by EU regulations for cereals and cereals products.

Data were presented at Conference in the International conference on Pollution, Environment, Health and Sustainable Development, Fez, Morocco, 8-12 November, 2010:

- Zizi S, Aissaoui A, Lyoussi B. Cadmium, mercury and lead in blood of women in Fez –Boulemane region (Morocco).
- Zizi S, Aissaoui A, Aazza I, Leszkovicz A, Dumont X; Bernard A, Lyoussi B Interaction between nephrotoxic metals and Ochratoxin A in Moroccan population from Fez Boulemane region.

Conclusions

The levels of Pb, Cd and Hg in blood were higher in Moroccan women than in most European countries (WP III:1). There were significant differences in blood metal levels between the rural, urban and industrial areas. However, there were no statistically significant associations between the metal concentrations in blood and excretions of proteins in urine, which are used to assess renal tubular damage. Further, in women, there were no associations between S-OTA and the kidney markers. Still, the OTA levels in bread were high.

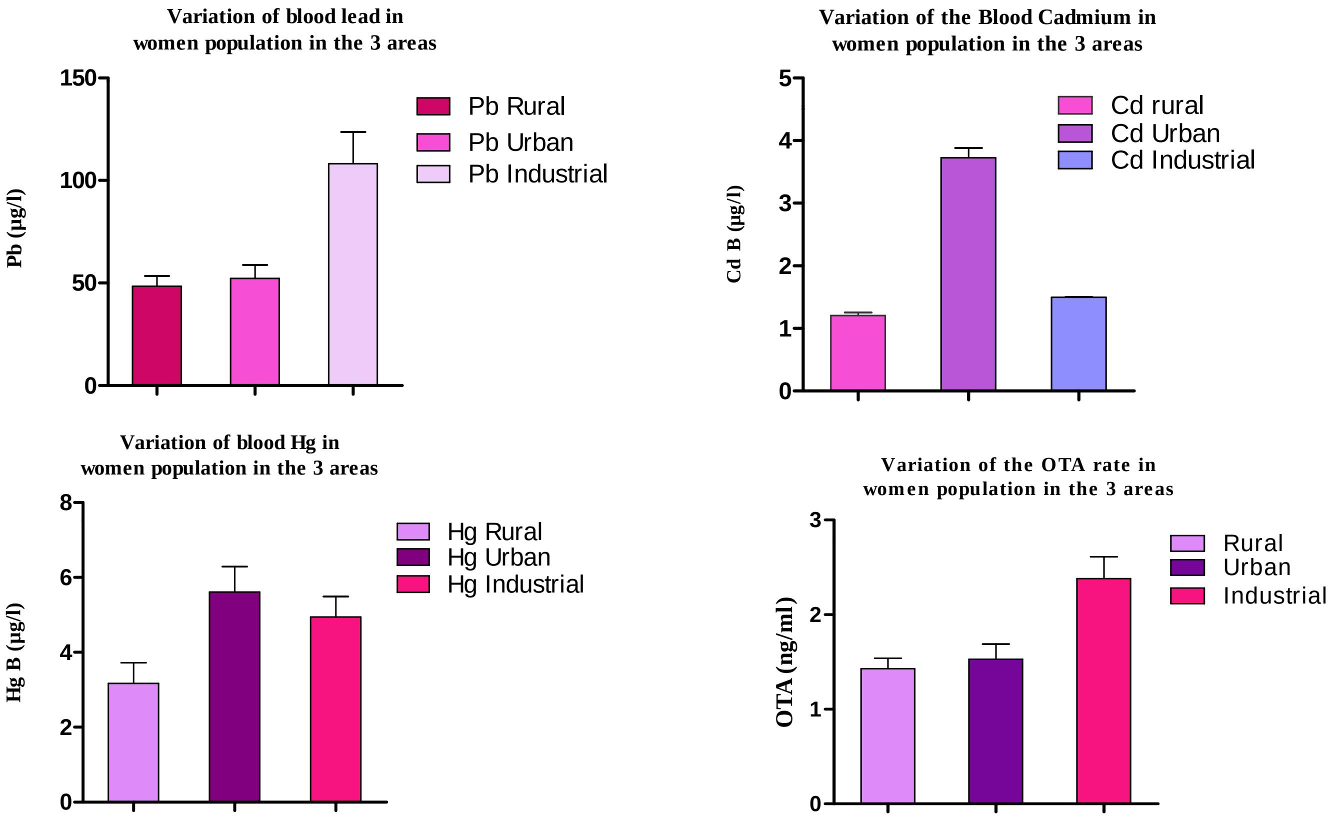

Figure 1. Concentration of lead (Pb), cadmium (Cd) and mercury (Hg) in whole blood, and Ochratoxin A (OTA) in blood serum in Moroccan women.

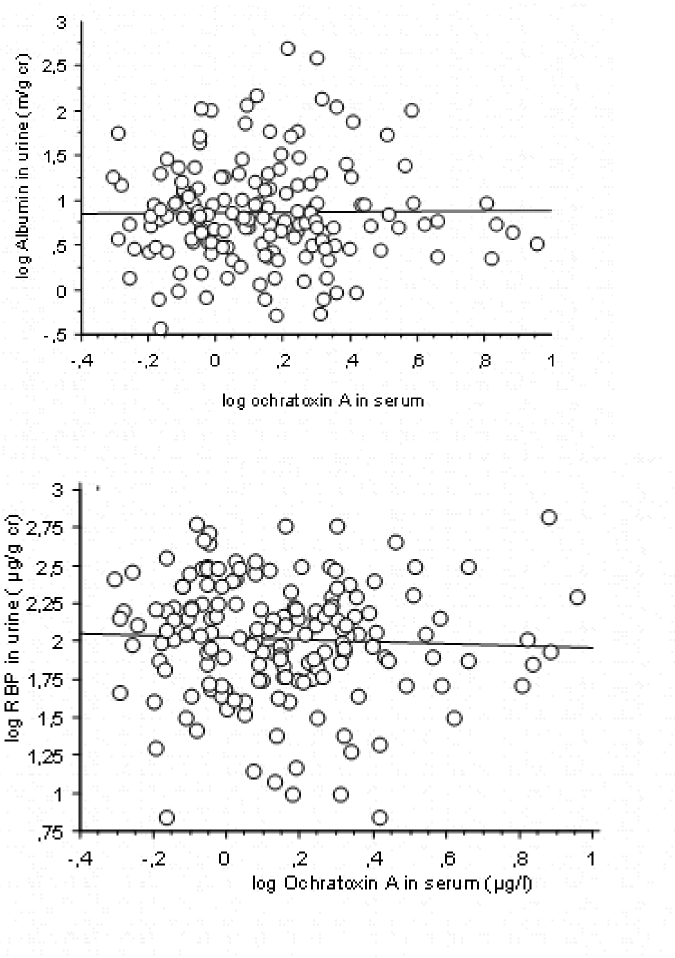

Figure 2. Concentrations of albumin (top) and retinol-binding protein (RBP; bottom) in urine (A) *vs.* Ochratoxin A in serum of Moroccan women.

Table 1. Ochratoxine A (OTA) level in cereal, bread, spices and olives.

Matrix	OTA (µg/Kg)
Wheat grain	0.53 (0.03-1.53)
Bread	4.30 (1.52-20)
Black olives	1.1 (0.5-3.2)
Cumin	0.03 (0.01-0.09)
Ginger	1.30 (0.07-6.4)

Over-all conclusions of Sub-Pillar II:3

Doubt is casted by the present results on the causality of associations between markers of renal tubular effects and low-level metals excretion. Risk assessment of Cd should also include other effects than nephrotoxicity, such as bone toxicity. The use of sample biobanks now facilitates prospective studies of long-term, clinically important kidney effects, and the first such study suggests an effect of lead - and maybe also cadmium - at low levels. The importance of a long-term view on kidney effects is further stressed by the study of previously highly exposed individuals, showing no or only limited recovery from tubular kidney effects.

In Bangladesh, urinary concentrations of metals and other elements differed largely with gender, age, tobacco use, socioeconomic and nutritional status. This should affect the assessment of the exposure and health impact of the mixed exposure to these potentially nephrotoxic elements. In Morocco, there was no evidence of interaction between Cd and the nephrotoxic mycotoxin OTA.

A biobank study of diabetes and Cd did not prove any strong association.

Section 1.3

Pillar III Where are the problems? (Pillar coordinator Ingvar A. Bergdahl)

When planning the PHIME project, we realized that in order to evaluate, at the EU level, the public-health consequences of exposures to metals, we would both need new information on both toxic effects (Pillars I and II) and on the general populations' exposure levels. Pollution by heavy metals varies between different European, and other, areas, and it changes over time. As regarded international comparisons with several countries, we could only find one study from the 1980's (Vahter and Slorach 1991), comparing metals in blood from school teachers living in different parts of the world. Information on time trends was available for lead (Pb) in blood, but not for cadmium (Cd) ormercury (Hg). We found it remarkable that so little was known about exposure levels, as this knowledge is fundamental for preventive actions – regionally, nationally and at the EU level. We were especially concerned about the Central and Eastern parts of Europe, because of the post-war establishment of heavy industries there.

At the same time, initiatives were taken to achieve Human Biomonitoring of environmental contaminants throughout Europe (leading to first the ESBIO and then the COPHES and DEMOCOPHES projects). The initial documents on such activities stressed the importance of 'learning by doing'.

In addition, new analytical developments had facilitated analysis of 'new' elements [e.g. those emitted from cars' catalytic converters: platinum (Pt), palladium (Pd) and rhodium (Rh)] in blood, and the 'old' element Pb could be determined in blood plasma, not only in whole blood and urine. We wanted to explore what implications this could have for human biomonitoring.

Against this background, we designed Pillar III, with two major parts: one focused on an international human biomonitoring study and follow-up of time trends (involving 12 Partners; WP III:1), the other on biomarkers of lead exposure and risk (five Partners; WP III:2).

WP III:1 Sources of exposure, geographical patterns and time trends: Exposure to elements in children and women

Summary description of WP objectives

- What are adequate sampling and analysis strategies for biomonitoring of exposures to some toxic elements (Pb, Cd, Hg, Pt, Pd, Rh) and their determinants?
- Which are the geographical patterns of exposure to these elements in different geographical areas, in particular in Central and Eastern Europe? For comparison, three non-European countres were included (Morocco, China and Ecuador).
- What are the time trends for Pb, Cd and Hg in Europe?

Contractors involved

UmU (WP leader Ingvar A. Bergdahl), IJS, Kau, NIPH, OIKON, RAPH, UMCL, Sosno, LU, IFA, PPSE, IHEP.

Degree to which the objectives were reached
Strategies for sampling and analysis – chemical as well as statistical – have been developed for biomonitoring of exposures to Cd, Pb, Hg, Pt, Pd and Rh in international studies. International and regional patterns have been identified in nine countries, and time trends of exposure to these elements have been evaluated in Sweden; data have been collected for Poland, Czech Republic and Slovakia, and are under processing.

Methodologies and approaches employed
Geographical pattern of exposure
We sampled blood from children and women in six European (Croatia, Czech Republic, Poland, Slovakia, Slovenia, and Sweden) and three non-European countries (China, Ecuador, and Morocco). Interview and questionnaire data were obtained. The blood was analyzed for Cd, Pb, and Hg, and in 248 of the women's samples also Pd, Pt, and Rh.

International comparisons were made between children living in cities in the nine countries. About 50 school children (6-14 years) from each city were recruited (totally 433) in 2007-2008. Likewise, a comparison was made between groups of about 50 women (46-62 years; totally 480) in 2006-2009. In addition, regional comparisons were made between children from urban, rural and industrial areas (totally 1,363).

The blood samples were analysed for Cd and Pb in one laboratory to exclude interlaboratory variances. The only exception was Hg analyses in children's blood, performed in two laboratories that had shown excellent agreement. In the regional comparisons, local laboratories were employed.

Cd and Pb were analysed buy inductively induced plasma mass spectrometry (ICP-MS), Pt, Pd and Rh by high resolution ICP-MS and Hg by either cold vapour atomic absorption spectrometry or neutron activation analysis.

Time trends of exposure
In the PHIME period 2006-2011, blood was sampled from about 500 children (age 7-8) from the town Landskrona in southern Sweden, in which there is a secondary lead smelter. Determinations of Pb and Cd were made by ICP-MS, of Hg by atomic emmision spectrometry. The information on these children was merged with determinations in about 3.500 children sampled 1978-2005 in Landskrona and the nearby town Trelleborg, in which there is no industrial metal emissions.

Information on B-Pb in children from Poland, Czech republic and Slovakia has been collected and put in a database together with the Swedish B-Pbs. Similarily, data on B-Cd and B-Hg in Polish and Czech children have been included.

Toxic effects of lead
In the Swedish children with B-Pb data from 1978-2007, information on later school performance (in grade 9, at age 16) was collected from Statistics Sweden, together with information on a series of socioeconomic conditions of the families. Further, information was collected on IQ, height and weight at examination of the "former boys" for military service (age 18-19), and on the fathers of all boys and girls at his examination as a conscript. Statistical analyses have been partly completed.

Achievements in relation to the state-of-the-art

Geographical patterns of exposure

The most striking observation was that European city children´s B-Cd and B-Pb varied only little between countries (geometric means: 0.11-0.17 and 13-19 µg/L, respectively; Hruba et al., submitted). Similarly, the concentrations differred only little in women's blood (Pawlas et al., submitted). B-Hg differed considerably between countries, due to varying tooth restoration practices and fish intakes (0.12-0.94 µg/L; Hruba et al., submitted; Pawlas et al., submitted).

In contrast, regional comparisons indicate that industrial 'hot spots' affect children´s exposure to Cd, Pb and Hg in certain countries, both in Europe and elsewhere (Hruba et al., manuscript). The levels in the non-European countries were generally higher.

As expected, dental amalgams and fish consumption influenced B-Hg, and gender influenced B-Pb. A new observation was that traffic intensity seemed to be a determinant for B-Cd. The metal concentrations were low from a risk perspective but the chosen non-European cities showed higher concentrations than the European ones.

Determinations Pt, Pd and Rh showed much lower concentrations than previously reported, with variations between women in different conutries (Rentschler et al., manuscript). There was a great analytical challenge, because of interaction between isotopes.

Time trends of exposure

The B-Pbs in children from Southern Sweden 1978-2011 showed a dramatic time pattern. Hence, there was a sharp decrease in the period 1978-1994, when Pb was eliminated from petrol (**Figure 1**; Strömberg et al. 2008; Stroh et al. 2009; Skerfving et al., manuscript; Nilsson et al., manuscript). However, there was also a decrease later, when there was no Pb in petrol, which indicates an effect of petrol Pb, which disappeares only slowly. A dcrease of B-Pb was also seen in women living in the north of Sweden (Wennberg et al., to be published). B-Pb data in children from Poland (WP I:7, and additional information), the Czech Republic and Slovakia are under analysis.

As to B-Cd in children from southern Sweden, there has been no obvious decrease in the period 1986-2011 (**Figure 2**; Skerfving et al., manuscript). A similar pattern was seen in women from northern Sweden (Wennberg et al., to be published). B-Cd data from Poland and the Czech Republic are under analysis.

As to B-Hg in children from Southern Sweden, there has been a decrease in the period 1990-2011 (**Figure 3**; Skerfving et al., to be published). A similar pattern was seen in women from northern Sweden (Wennberg et al., to be published). B-Hg data from Poland and the Czech Republic are under analysis.

Toxic effects of lead

In the Swedish children with B-Pb data from 1978-2007, the mean B-Pb was as low as 37.1 (median 33, range 5.6-244) µg/L, with - as said above - a six-fold decrease over the time period (**Figure 1**). There were statistically significant negative associations between later school performance (in grade 9, at age 16) and earlier B-Pb. Similar statistically significant associations were found for IQ, height and weight at examination of the boys as young men for military service (age 18-19) and earlier B-Pb. Further statistical analyses are under way.

Conclusions
There is no general elevated exposure to Cd or Pb in any of the studied European countries, but industrial 'hot spots' that significantly affect children's exposure can be identified in some countries, e.g. Poland, Slovakia and the Czech Republic. For Hg, there was a variation between European contries, which was explained by variation in fish consumption and dental practices. In general, higher levels were found in China, Ecuador and Morocco.

For the "catalytic converter elements" Pt, Pd and Rh, much lower levels were found than reported earlier. This is because of the great analytical challenge of these elements.

The experience of these studies tells that it is not advisable to rely only on national/local laboratories when performing international comparisons in biomonitoring studies, because the international variation in biological concentrations may be so small that differences between laboratories' performance cause a considerable bias of the results.

In Sweden, there has been a rapid decrease of B-Pb in children and women after the start of elimination of Pb from petrol in 1978, and the final disappearance of petrol Pb in 1994. There has also been a decrease of Hg exposure. No clear decrease of Cd exposure has occurred, which is a serious problem, in particular in perspective of the effects on the skeleton (Sub-pillar II:2 above). Time trend data on Pb, Cd and Hg from other areas of Europe are under analysis.

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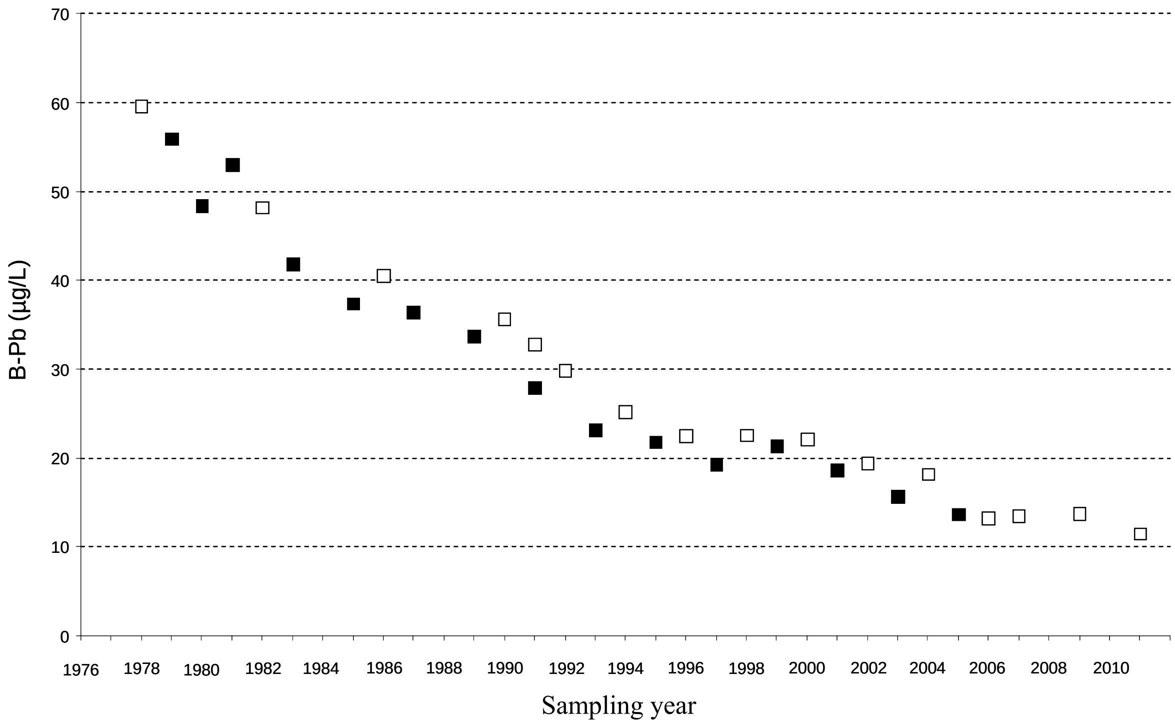

Figure 1. Lead concentration in blood (B-Pb, geometric means) in 4,050 children (mainly age 7-8) in southern Sweden [towns Landskrona (open symbols) and Trelleborg (closed)] examined 1978-2011.

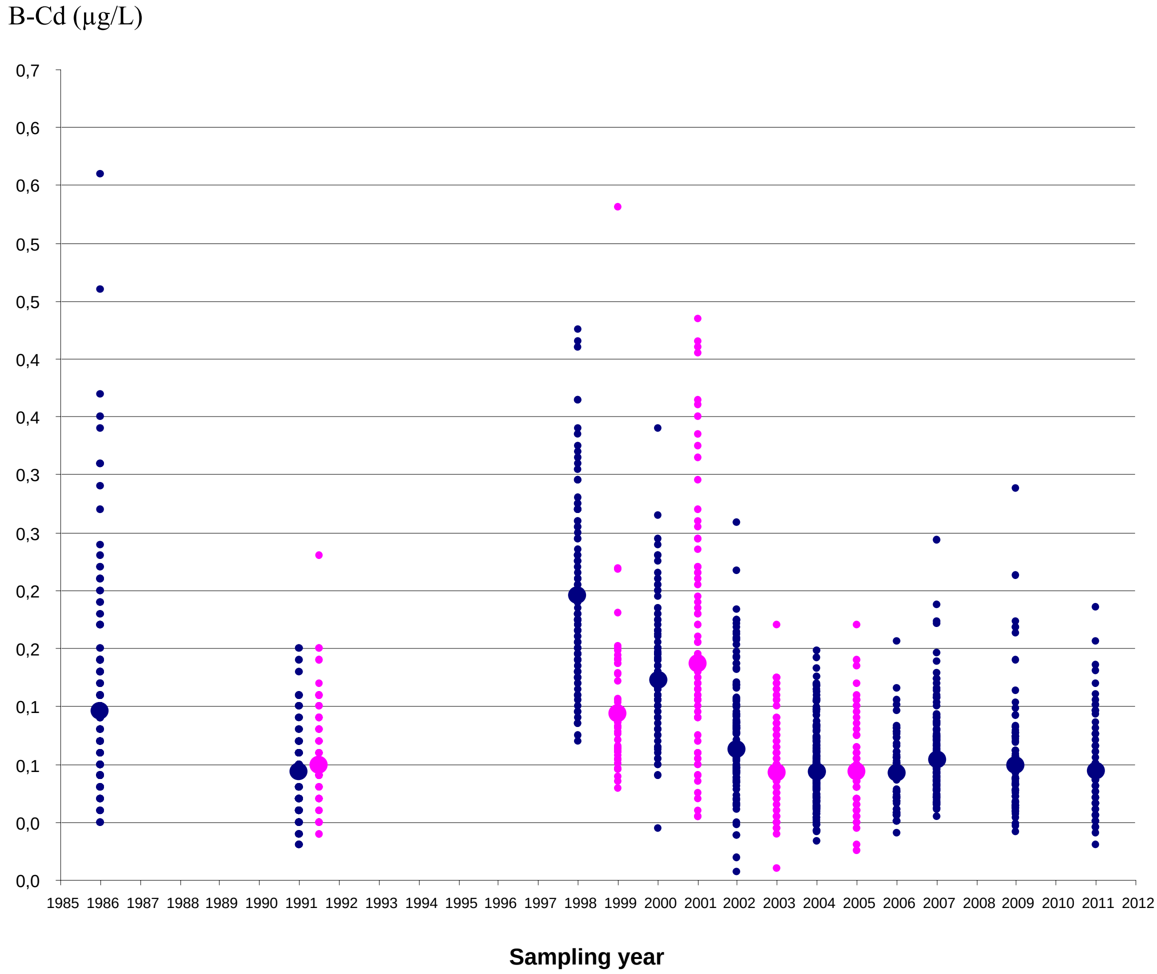

Figure 2. Cadmium concentration in blood (B-Cd, geometric means and individual measurements) in 1,521 children (mainly age 7-8) in southern Sweden [towns Landskrona (black) and Trelleborg (red)] examined 1986-2011.

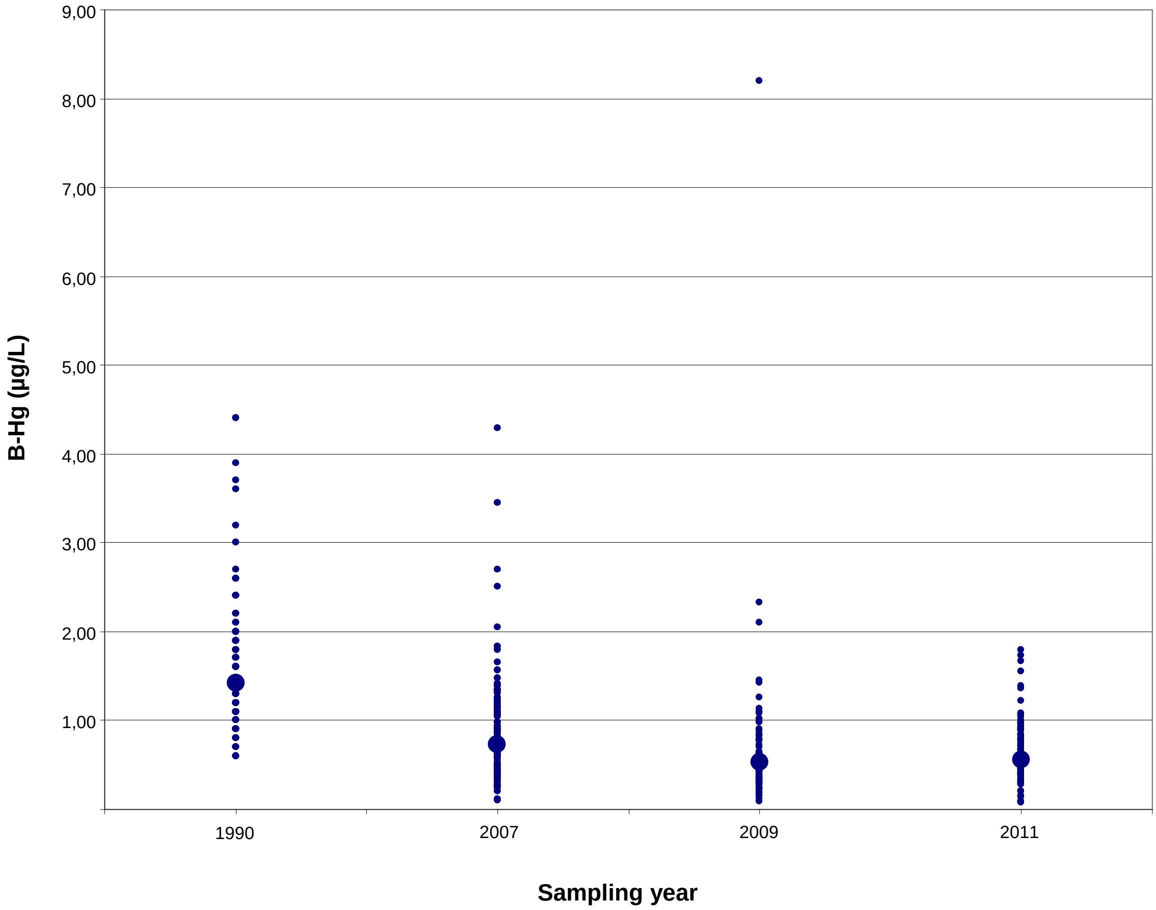

Figure 3. Mercury concentration in blood (B-Hg, geometric means and individual measurements) in 1,169 children (mainly age 7-8) in the southern Sweden town of Landskrona) examined 1990-2011.

WP III:2 Biomarkers of lead exposure and risk

Summary description of WP objectives
Lead in blood (B-Pb) is the major tool for biomonioring of Pb exposure and risks. However, it has serious limitations, mainly due to a saturation at high exposures, which limits its usefulness.

The main objective of WP III:2 was to test the hypothesis that Pb in plasma (P-Pb) is a better biomarker. The method for sampling of blood and determination of P-Pb should be improved. Also, the meaning of P-Pb in lead workers and controls should be tested, as the relationship between P-Pb and toxic effects would be described.

Moreover, the advantages (assumed recti-linearity) of P-Pb would be used to solve the issue of whether genetic traits modify the relationship between exposure and risk. Because of variations in gene frequencies between populations, studies should be performed in both China and Europe. Such differences may limit the possibility to merge information from different populations in risk assessment activities, which has been the usual way.

Contractors involved
Sosno (WP leader Krystyna Pawlas), LU, Fudan, NIPH, UmU.

Degree to which the objectives were reached
Almost all the planned activities have been finalized. Statistical analysis of data from Poland and the Czech Republic is under way; the models have been established in the finished analyses of the Chinese data.

Methodologies and approaches employed
Large cohorts of Pb workers and controls have been extensively examined in field work in China (biomarkers of exposure and risk; function of peripheral nervous system=PNS and kidneys, haem synthesis), Poland (biomarkers; function of PNS, hearing, posture, kidneys) and the Czech Republic (biomarkers; renal function, haem synthesis). Genotyping for two genes [δ-aminolevulinic acid dehydratase (*ALAD*; two single nucleotide polymorhisms=SNPs) and vitamin D receptor (*VDR*; threeSNPs) genes] has been performed. Chemical analyses of biomarkers of exposure and risk have been made [whole blood (B-Pb), urinary (U-Pb) and plasma (P-Pb) Pb]. In particular, the method for determination of P-Pb has been refined. Markers of kidney function and haem synthesis have been analysed. Databases have been established and analysed multivariately, fully (China) or partly (Poland, Czech Republic). Statistical analysis of the Chinese data has been finalized, and manuscripts have been submitted. Analysis of Polish and Czech data is under way.

Achievements in relation to the state-of-the-art

- After developmental work regarding sampling equipment and analytical procedure, good performance was obtained as regards determinations of P-Pb.
- The ratio between Pb biomarkers in Pb workers and unexposed controls was greater for P-Pb than for U-Pb and B-Pb.
- There were close associations between P-Pb, B-Pb and U-Pb.
- However, there was a non-linear relationship between B-Pb and P-Pb.
- In the Chinese Pb workers, *ALAD* (*Msp*I) genotype modified B-Pb and U-Pb in lead workers, while P-Pb was not affected. It seems that there is a genetically determined

difference in saturation of Pb binding to ALAD protein in blood (the major binding site).

- *VDR* genotype (three SNPs) did not affect B-Pb, U-Pb or P-Pb, which is opposite to earlier results, and needs further penetration.
- There were negative effects of Pb exposure on PNS in motor and sensory nerves (ulnar, median and peroneal nerves), renal function (albumin, $ß_2$-microglobulin and *N*-acetyl-ß-D-glucosaminidase in urine) and heam synthesis (zinc protoporphyrin and haemoglobin in blood and δ-aminolevulinic acid in urine).
- P-Pb displayed closer association than B-Pb and U-Pb with effects on PNS and renal functions, and haem synthesis.
- *ALAD MspI* genotype modified the relationship between B-Pb and U-Pb, on the one hand, and effects on PNS functions, on the other. *ALAD*2 carriers seemed to be less sensitive.
- *ALAD* genotype also modified the relationships between P-Pb, on the one hand, and markers of effects on renal function and haem synthesis, on the other.
- *VDR* polymorphism did not modify these relationships (PNS and renal function or haem synthesis). This has been discussed during the last decade.
- Among the Chinese Pb workers and controls, the frequency of *ALAD*2 carriers was low. However, the frequency in the workers was higher than among unexposed controls. There were no differences in *VDR* genotypes.

Conclusions

- It is possible to determine P-Pb with sufficient analytical quality, using inductively-induced mass spectrometry (ICP-MS), if Pb-free sampling tubes are used, and if haemolysis is avoided. This means an important development of the method, and makes P-Pb an attractive possibility for biomonitoring of exposure to - and risk of - lead exposure. This is important, considering the limitations of B-Pb at high exposure.
- The P-Pb levels in the Chinese Pb workers and unexposed controls were low (as compared to B-Pb), but in accordance with earlier reports.
- In accordance with the hypothesis, P-Pb seems to be recti-linearily related to exposure, making it a better biomarker of high exposure to Pb than B-Pb, which gets saturated. This is of importance, since it limits the usefulness of B-Pb (the major tool presently used for lead biomonitoring) at high exposure/risk. P-Pb was also better than U-Pb, which varies over time. This shows that P-Pb has advantages over B-Pb and U-Pb as a biomarker of exposure.
- The *ALAD* (*Msp*I) gene frequencies were similar to those reported earlier in Asian populations, which is much lower tha in Europeans. The *VDR* gene frequencies were in accordance with earlier reports.
- B-Pb and U-Pb (but not P-Pb) are modified by *ALAD* genotype. This means that different populations, with varying gene frequencies, will differ in B-Pb at a certain exposure. This solves a dispute on the effect of *ALAD* interaction, which has been around for two decades. It must be considered in risk assessment, where B-Pb is the generally used biomarker of risk, and where information from different populations is often merged. Population-specific risk assessments may be needed.
- P-Pb displayed closer association than B-Pb and U-Pb with effects on PNS and renal functions, and haem synthesis. This novel finding shows the adavantage of P-Pb over B-Pb in assessment of toxicity.

- *ALAD* genotype modifies the relationship between biomarkers of exposure and risk (B-Pb, U-Pb, P-Pb), and effects on the PNS (which has not been shown earlier) and effects on renal function and haem synthesis (varying results earlier). This modification is not due to a simple shift in the exposure-response curves (which is a possible interpretation of earlier results), but seems to be a true difference in sensitivity, meaning that *ALAD*1-1 homozygotes are more sensitive to Pb toxicity than *ALAD*2 carriers, which is a novel conclusion, of importance for the risk assessment in different populations.
- There was a selection for *ALAD*2 carriers in Chinese Pb workers, which may indicate a selective quitting by *ALAD*1-1 homozygotes. A selection by employment time has earlier been seen. However, such a healthy worker selection may work be differently in factories with regular screening for B-Pb, where *ALAD*2 carriers may quit because they obtain higher B-Pb at certain exposure, and in factories without such screening, where *ALAD*1-1 genotypes may quit because of Pb toxicity. Indeed, paresthesias were more common among *ALAD*2-carrying Pb workers, which may be a recognizable side effect of the exposure, caused by the sensitivity of the PNS, and might induce such selective quitting.
- *VDR* genotype, contrary to some earlier reports, did not affect the biomarkers of exposure and risk, in spite of the fact that Pb strongly interacts with the calcium metabolism, in which VDR is a key player.
- The results in the Chinese Pb workers differed to somewhat from those in Polish children (WP 1:7) with much lower Pb exposure, in whom one *ALAD Msp*I genotype did not affect B-Pb, while *ALAD Rsa* did, as did two of the three *VDR* genotypes. This might be due to different gene frequencies, or the lower exposure. Interestingly, in the Polish children, *ALAD Rsa* genotype seemed to modify the negative effect of Pb on cognitive functions (IQ), which is in accordance with the modification by *ALAD* genotype of the toxic effects on PNS in the Chinese Pb workers.

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Global deliverables of Pillar III

The results can have a number of implications. The scientific dimensions will be discussed elsewhere, but here are some issues that could concern EU initiatives or policy.

Identification and remediation of metal-contaminated sites can decrease harmful exposure. The international study indicates that Cd and Pb pollution is not a general problem in certain countries, but rather a problem in specific areas. As both Cd and Pb exposures are of health concern in the general population (PHIME Pillars I and II), a doubled exposure among children is significant and should be prevented, if possible. Since the sources are often known and it is relatively uncomplicated to evaluate the exposure, a programme aiming at improvement of the worst residential areas could have a significant impact.

As was clear from the opinion of EFSA 2009 on Cd in food, there is concern for the health effect of the Cd intake in Europe today. Results from PHIME (Sub-Pillar II:2) on bone effects suggest that public-health effects may be even worse. It is, thus, worrying to note that cadmium concentrations do not show any sign of decrease. The intake of Cd is not something that the individual can do much to decrease; much of it comes from "healthy" foods (whole - grain cereals and vegetables) that we should continue to eat. Therefore, it is the producers and society that must take actions to decrease the Cd contamination of agricultural soil.

Large-scale environmental Human Biomonitoring studies should make both international and regional comparisons of exposure possible. However, the unexpectedly low international variation for Cd and Pb caused problems. Originally, we employed different laboratories in different countries for the analyses but quality control schemes at these concentrations can only assure that the laboratory results differ less than ±20%. In the case of Cd and Pb, that was not sufficient. Therefore, we had to re-analyzed all urban samples in one single laboratory, in order to facilitate an international comparison. This is a hard lesson; we hope that other projects will not have to learn themselves.

While Pb exposure has decreased, so have also the ‘safe levels’. EFSA could in 2010 not come to an opinion on a safe intake of Pb. Effects on cognitive functions (WP I:7 and WP III:1), hearing and posture (WP I:7) of Pb are observed at B-Pb ≤ 50 µg/L in blood, but there appears to be no threshold, implying that the effects occur in the general population. In addition, one PHIME study (WP II:3:3) suggests long-term effects in the form of increase risk for kidney failure at the general population’s Pb exposure levels. Therefore, there are reasons to decrease Pb exposure further, despite the success of abolishment of Pb in petrol. What can we then do to decrease Pb exposure? The scientific answer is that we do not know. Very little efforts have been made to identify the most influential sources and pathways of Pb exposure after the abolishment of Pb in petrol. Research should, thus, try to identify these sources and pathways. Many researchers would regard this as ‘old fashioned’, but here the EU could be pro-active through the framework programme.

Plasma-Pb concentration may be slightly better than whole blood for biomonitoring of Pb, but it is not so much better that it can replace blood in biomonitoring of workers or in environmental epidemiology – yet. We see no reason to suggest a replacement of B-Pb with P-Pb, neither in Pb exposure monitoring within occupational health services, nor in environmental epidemiology. However, with the indications that P-Pb is stronger correlated to health effects in Pb workers, despite a poorer precision, we think that P-Pb should be a measure included in parallel with B-Pb in studies of exposure and effects of Pb.

Section 1.4
Pillar IV Possible solutions of the problems (Pillar co-ordinator Michael Broberg Palmgren)

Summary of Pillar goals

Plants constitute a major source of heavy metal intake in human populations. Plants are the basis of the food chain and accumulate a range of metals from the soil. This problem has two sides. On the one hand, we would like to avoid toxic concentrations of heavy metals in our food and feed and on the other hand, humans and other animals need supply of 16 minerals, including some heavy metals and transition elements, through their diet. The challenge here is to produce plants containing adequate amounts of essential minerals like zinc (Zn) and iron (Fe) in the edible parts, and avoiding toxic levels of harmful elements like cadmium (Cd) and mercury (Hg).

Objectives and integration within PHIME

Whereas Pillars I, II and III did supply information on the size, determinants, geographical patterns and expected development over time of existing and expected metal-associated health problems, as well as beneficial interactions between essential elements and other constituents of foods, Pillar IV has explored possibilities to use this information for prevention.

One obvious aspect has been to supply scientifically-based information to national, EU and international bodies, which can use it for decision making of political and economical character, e.g., measures to reduce pollution and provide dietary recommendations to vulnerable groups. This has been an important part of the WP C:9 dealing with dissemination.

Another aspect has been extensively explored: Possibilities to reduce the uptake of toxic elements into plants in order to reduce the exposure from vegetable foods, as well as the incorporation of toxic elements into the food chain. In a first step, a series of model plants (including barley, tomato and tobacco) has been studied, but the principles are common for many vegetables, thus being of a general nature. These studies were made possible through cutting-edge plant physiology, employing molecular, biochemical and electrophysiological approaches.

Another important possibility, that has been further explored, is the possibility of increasing elements, which interact with the toxic ones, and which are deficient in food from different Member States. As a first step, the uptake and transport of Zn was studied in model plants. It is estimated that one third of the world’s population suffer from inadequate intake of Zn.

Heavy metal uptake in crop plants: The prime entry point in the human food chain

Often, agricultural and natural soils are rich in heavy metals, either as a result of pollution or because of the geological origin of the soil. Thus, in order to reduce harmful heavy metal intake in humans, it is essential to reduce the content of these metals in crop plants. The cereals are by far the most important food source, providing the food basis of the major part of the world’s population.

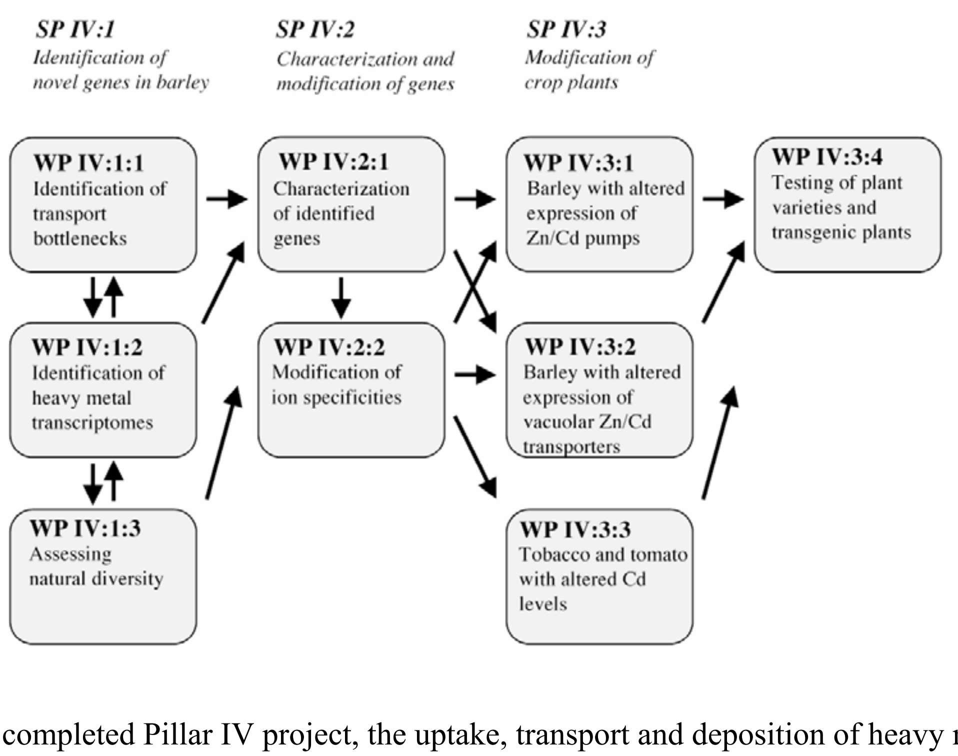
In Pillar IV, cross-disciplinary research has been implemented to reduce harmful heavy metal intake into the human food chain. In FP5, the European Commission funded the project "Metallophytes" that addressed cleansing of polluted soils by heavy metal-accumulating plants. Several of the scientists in Pillar IV of PHIME participated in this project. Thus, personal networks and routes for exchange of knowledge and tools were already in place and could be utilized within the PHIME project.

At the onset of PHIME, very little was known concerning heavy-metal handling by crop plants. The work in FP5 focused on model plants such as *Arabidopsis thaliana*, the genome of which is fully sequenced. This was important for elucidating many of the basic processes associated with plant heavy-metal handling. In the now completed FP6 project, the knowledge obtained in FP5 was related to that of human toxicology to identify, for the first time, markers for the breeding of healthier crop plants with respect to their heavy-metal contents.

In plants, a complex and well-regulated metal homeostasis system orchestrates transport and chelation events in all plant organs and cell types to ensure the adequate supply of nutrient transition metals to their target sites. This system involves metal mobilisation in the soil, root metal uptake, root-to-shoot translocation, metal distribution between cell types and organs, storage, and remobilisation of metal stores. The specific architecture of the metal homeostasis systems, represented by differential expression of metal homeostasis genes, determines the metal accumulation profile in the different tissues, and has been shown to exhibit significant natural diversity.

In some species and tissues the homeostasis system can accommodate heavy metals in concentrations up to 1,000 fold that found in other species/tissues, via a range of mechanisms such as sequestering to intracellular compartments and binding to particular polypeptides or other organic compounds. When edible parts from such plants are consumed, the bioavailability of heavy metals in the human digestive tract is highly dependent on how they are complexed with organic or other inorganic compounds.

Fig. 1. Overview of workpackages in Pillar IV



In the now completed Pillar IV project, the uptake, transport and deposition of heavy metals in barley were addressed: barley is a representative of the small-grained cereals that comprise the major food source for humans, and is a close relative of wheat. Additionally, the most promising genes were introduced into tomato and tobacco, representatives of fruit and leaf crops. By integrating state of the art technologies in plant physiology, molecular biology and genetics, certain plant genes were identified, characterised and manipulated. The genes of concern were those involved in i)avoidance of harmful metals; or ii) inclusion of metals of nutritional concern in the grain and in leaves. The focus was on Cd, Hg, Zn and Mn.

The toxicity of Cd and Hg is well known. Cd and Hg are not considered essential metal elements for humans but there is increasing concern about human health risks. An element such as Zn reduce the toxicity of heavy metals such as Cd, Pb and Hg. These metals interact with toxic elements, e.g., by inducing metallothionein biosynthesis. There is an increasing awareness that Zn may be one of the most important minerals in human nutrition as it is an indispensable co-factor for about one thousand transcription factors and more than three hundred enzymes in humans. Mn is likewise an essential co-factor for several enzymes in plants and animals. However, both Mn and Zn are toxic at higher concentrations. Moreover, the concentration differences between beneficial and toxic Mn are relatively narrow. Some of these issues were further addressed in a horizontal WP C:4, devoted to nutritional toxicology.

Objectives of Pillar IV

Specifically, the Pillar intended to investigate what happens to Cd, Hg, Zn and Mn once crop plants absorb these elements. Important questions that remained to be solved were:

i) Where are the cellular bottlenecks for their absorption and deposition into the grain?

ii) What are the principal immobilisation mechanisms, whether they are through binding or sequestration, and how do they affect bioavailability?

iii) What molecular markers can assist breeding, to optimise the metal content of plants?

Sub-Pillar IV:1 Identification of transport barriers and heavy metal related genes in barley

Summary description of objectives

Background

The general aim of SP IV:1 was to elucidate the bottlenecks for transport and deposition of heavy metals in the cereal grain and to explore the genetic variability related to heavy metal handling of barley. This knowledge is essential for future creation of new cultivars with improved heavy metal management.

Metal transport and deposition in the cereal grain is of high complexity. Solutes unloaded from the phloem move symplastically or apoplastically through a series of tissues, until they are unloaded in the grain. Two layers of so-called transfer cells characterised by highly convoluted plasma membranes, indicating a high transport potential are of particular relevance for the final transport into the grain.

At the onset of the Project, very little was known concerning the molecular nature of these barriers. It was expected to identify four different depositories for heavy metals in the grain: embryo, endosperm, aleurone and the surrounding maternally-derived tissues, also referred to as the seed coat. For human consumption, rice and wheat grains are generally processed by milling to obtain pure endosperm, a bran fraction consisting of the seed coat and the aleurone, and an embryo fraction.

Other cereals are consumed in a largely unprocessed form. Bran fractions from various cereals are popular dietary supplements, as they contain health-promoting fibres. It is thus important to know the concentration, speciation and bioavailability of metals in each of these grain fractions.

It is apparent that the primary depository for Zn and Mn is vacuoles in the aleurone layer, where the metals are complexed with phytic acid. This compound readily binds Zn, Ni, Co, Mn, Ca and Fe (with decreasing affinity), as well as K. The second most important depository for Zn and Mn is most likely the embryo, due to the importance of these metals as co-factors for a range of enzymes, as well as transcriptional activators (Zn). Very little was known at the onset of the Project about the deposition of Cd, Hg, Zn, Mn and Se in different tissues in the cereal grain, in particular the endosperm, which is the tissue of most relevance for human consumption.

Objectives of Sub-Pillar IV:1

i) To identify the bottlenecks in barley for the transport of the elements Cd, Hg, Zn, Mn and Se, the concentration and speciation of the five elements were measured at different transport steps along the pathway from roots to shoots, as well as in the different tissues of the barley grain. Barley plants were grown on three types of reference soil: normal, mildly polluted and Zn-deficient (calcareous) soil;

ii) To determine the size and the nature of the transcriptome related to these elements;

iii) To explore whether the transcriptome could be used as a primary tool to identify genes encoding key proteins determining transport and deposition of the five elements;

iv) To investigate the potential of genetic variability in barley for future marker-assisted breeding, aiming at improved heavy metal related properties;

v) To capitalise on natural diversity to identify novel heavy-metal related genetic markers;

vi) To initiate work to test whether these markers could be employed for the breeding of toxicologically enhanced varieties of crops.

WP IV:1:1 Identification of transport barriers essential for the deposition of heavy metals

Contractors involved

KVL (WP leader), UBT, York, Flakke, IETU.

Degree to which the objectives have been reached

The objectives have been achieved.

Methodologies and approaches employed

Rationale

In order to characterise metal loading into the grain and the transport and deposition within the grain, it was essential to know the exact concentration of the different metals along the transport pathway. Therefore, heavy-metal accumulation had to be determined at the organ, tissue and cellular levels along the transport pathway from roots to grains in barley.

Experimentation

High-resolution liquid chromatography hyphenated with inductively coupled plasma mass spectrometry (ICP-MS) was employed for cell specific determination of heavy-metal contents. This instrumentation has accuracy and detection limits down to femtogram levels. The main components of individual barley grain tissues were fractionated in proteins, carbohydrates (incl. phytate andinositol phosphates) and secondary chemical compounds (incl. polyphenols, flavonoids and ascorbic acid), separated according to size, charge and hydrophobicity. LC-ESI-Q-TOF-MS was employed for sensitive detection and quantification of known and suspected metal ligands. Also, metabolome changes were monitored upon metal exposure and deficiency.

Interactions between Partners and WPs

Right from the start of the Project, a close interaction was initiated between partners KVL, LIP, York, Flakke and IETU, with respect to selection of barley genotypes and establishment of plant growth conditions and metal supply rates. The metal supply rates were chosen so that they spanned the variations occurring in uncontaminated and contaminated agricultural soil at different pH values. The interactions between Partners also served as a means to ensure a close co-ordination with WP IV:3:1, assessing natural diversity among barley genotypes, and WPs IV:1:2 and IV:2:1, focusing on identification and characterisation of genes involved in metal transport. Methods for decontamination of plant samples, particularly soil-grown roots, prior to analysis, were determined. Experiences with different protocols and equipment to dissect grain into bran, aleuron, endosperm and embryo prior to metal analysis were exchanged and a joint protocol agreed upon. Special emphasis was put to methods for extractions of tissues prior to analysis of metal speciation (complexes with peptides, proteins, etc.). A selection of tissue samples from all Partners were analysed both by Partners KVL and LIP in order to compare the performance of the ICP-MS at KVL and the AAS at LIP. In this work, appropriate reference standards from the National Institute of Standards and Technology (NIST) were included.

Achievements in relation to state-of-the-art

Quantitative multi-elemental analysis by ICP-MS depends on a complete digestion of solid samples. However, fast and thorough sample digestion is a challenging analytical task, which constitutes a bottleneck in modern multi-elemental analysis. Additional obstacles may be that sample quantities are limited and elemental concentrations low. In such cases, digestion in small volumes with minimum dilution and contamination is required in order to obtain high accuracy data.

We developed a micro-scaled microwave digestion procedure, and optimized it for accurate elemental profiling of plant materials (1-20 mg dry weight) (Hansen et al. 2009). A commercially available 64-position rotor with 5 mL disposable glass vials, originally designed for microwave-based parallel organic synthesis, was used as a platform for the digestion. The method developed constitutes a valuable tool for screening of mutants and transformants. In addition, the method facilitates studies of the distribution of essential trace elements between and within plant organs, which is relevant for, e.g., breeding programmes aiming at improvement of the micronutrient density in edible plant parts. Compared to existing vial-in-vial systems, the new method developed represents a significant methodological advancement in terms of higher capacity, reduced labour consumption, lower material costs, less contamination and, as a consequence, improved analytical accuracy following micro-scaled digestion of plant samples.

Barley grains were fractionated into awns, embryo, bran and endosperm and analysed for Fe and Zn. Simultaneously, phosphorus (P) and sulfur (S) were determined, since these elements are major constituents of phytic acid and proteins, respectively, compounds which are potentially involved in Fe and Zn binding. A novel analytical method was developed in which oxygen was added to the octopole reaction cell of the ICP-MS. This approach greatly improved the sensitivity of sulfur, measured as $^{48}SO^{+}$. Simultaneously, Fe was measured as $^{72}FeO^{+}$, P as $^{47}PO^{+}$, and Zn as $^{66}Zn^{+}$, enabling sensitive and simultaneous analysis of these four elements. The highest concentrations of Zn, Fe, S and P were found in the bran and embryo fractions. Further analysis of the embryo using SEC-ICP-MS revealed that the speciation of Fe and Zn differed. The majority of Fe co-eluted with P as a species with the apparent mass of 12.3 kDa, whereas the majority of Zn co-eluted with S as a 3 kDa species, devoid of any co-eluting P. Subsequent ion pairing chromatography of the Fe/P peak showed that phytic acid [myo-inositol-1,2,3,4,5,6-hexakisphosphate: IP(6)] was the main Fe binding ligand, with the stoichiometry Fe(4)[IP(6)](18). When incubating the embryo tissue with phytase, the enzyme responsible for degradation of phytic acid, the extraction efficiency of both Fe and P was doubled, whereas that of Zn and S was unaffected. Protein degradation on the other hand, using protease XIV, boosted the extraction of Zn and S, but not that of Fe and P. It was concluded that Fe and Zn have a different speciation in cereal grain tissues; Zn appears to be mainly bound to peptides, while Fe is mainly associated with phytic acid (Persson et al. 2009).

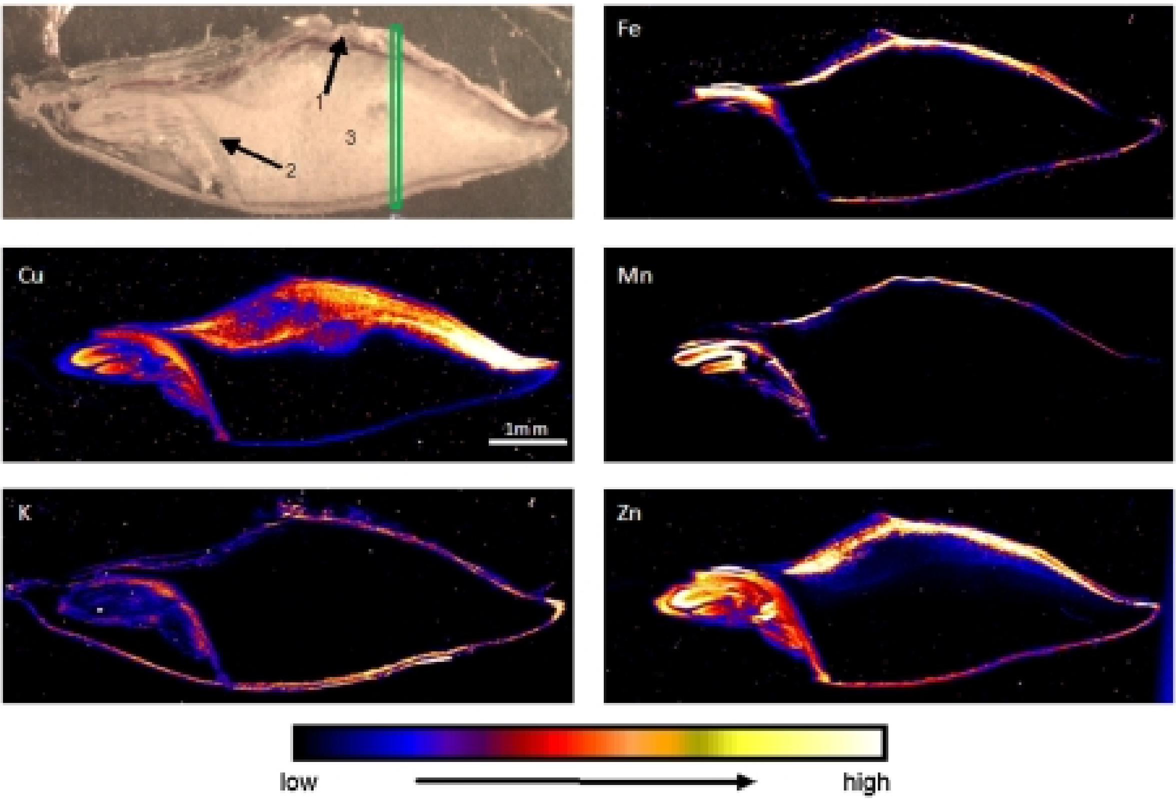


Figure 2. XRF elemental maps of a longitudinal section of a barley sample (ventral side on top). The colour scale represents different concentrations, with black and white corresponding to the lowest and highest concentration, respectively. The box in the microphotograph

corresponds to the area used to obtain the line scan presented in Fig. 3. The arrows indicate: 1, awns, testa, and aleurone layer; 2, scutellum; 3, endosperm. From Lombi et al.(2011).

High-definition synchrotron X-ray fluorescence was used to investigate the distribution and association of essential elements in barley grain at the micro scale (Lombi et al. 2011). Micronutrient distribution within the scutellum and the embryo was shown to be highly variable between elements in relation to various morphological features. In the rest of the grain, the distribution of some elements such as Cu and Zn was not limited to the aleurone layer but extended into the endosperm. This pattern of distribution was less marked in the case of Fe and, in particular, Mn. A significant difference in element distribution was also found between the ventral and dorsal part of the grains. The correlation between the elements was not consistent between and within tissues, indicating that the transport and storage of elements is highly regulated. The complexity of the spatial distribution and associations has important implications for improving the nutritional content of cereal crops such as barley.

The multielemental composition of organic and conventional winter wheat, spring barley, faba bean, and potato was analyzed with inductively coupled plasma-optical emission spectrometry (ICP-OES) and -mass spectrometry (ICP-MS). The crops were cultivated in two years at three geographically different field locations, each accommodating one conventional and two organic cropping systems. The conventional system produced the highest harvest yields for all crops except the nitrogen-fixing faba bean, whereas the dry matter content of each crop was similar across systems. No systematic differences between organic and conventional crops were found in the content of essential plant nutrients when statistically analyzed individually. However, chemometric analysis of multielemental fingerprints comprising up to 14 elements allowed discrimination. The discrimination power was further enhanced by analysis of up to 25 elements derived from semiquantitative ICP-MS. It was concluded that multielemental fingerprinting with semiquantitative ICP-MS and chemometrics has the potential to enable authentication of organic crops (Laursen et al. 2011).

Conclusions

The transport and storage of elements is highly regulated. The complexity of the spatial distribution and associations has important implications for improving the nutritional content of cereal crops such as barley. Multielemental fingerprinting with semiquantitative ICP-MS and chemometrics has the potential to enable authentication of organic crops.

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WP IV:1:2 Identification of the transcriptome correlated with heavy metal transport

Summary of WP objectives

Rationale

As a first step toward identifying genes essential for heavy-metal handling in barley, it was intended to characterize the heavy metal-related transcriptome of this plant. Some tissues could be obtained by conventional dissections, while in other cases this approach was not feasible. In these cases state-of-the-art technologies were employed to solve these problems.

Objectives

With barley as a model plant, the focus of WP IV:1:2 has been:

- To identify genes in the individual tissues of the unloading zone and the different tissues of the barley grain of importance for transport and deposition of heavy metals, like Zn, Cu, Fe, Se, Mn, Hg and Cd, via transcriptome analyses.
- To identify genes of particular importance for heavy-metal transport and deposition that could be subjected to focused studies by i*n vitro* mutagenesis, complementation in microorganisms and transformation of barley.

Contractors involved

Flakke (WP leader) KVL, UBT, York, MPS, UZH.

Degree to which the objectives have been reached

Most objectives have been reached during the project period. However, the establishment of a 454 sequencing facility was delayed; now the analysis is ongoing.

Methodologies and approaches employed

Experimentation

For gene identification, the rich source of barley and wheat EST sequences (350.000 and 500.000, respectively), as well as the rice genomic sequence, were used. When appropriate, commercially available 22k barley and 61k wheat oligonuceotide arrays were used. Due to the very high homology between barley and wheat gene sequences it was assumed that barley and wheat cDNA sequences could be used interchangeably. In addition to available gene arrays, a barley cDNA microarray was assembled that contained all major gene families of genes encoding transporters (e.g., LCT1, ZIP, Nramp, ABC, CDF type, P_{1B} ATPases, and YSL), as well as genes with putative functions in deposition (e.g., genes involved in phytic acid deposition) and neutralisation (e.g., metallothioneins, phytochelatin synthases and nicotianamine synthases).

Gene arrays were utilised to monitor differences in the gene expression profile following foliar applications of the five metals or addition of metals to the culture medium in hydroponic cultures. There was also a range of barley genotypes available that differ in their capacity to tolerate and accumulate the five metals. Through these approaches it was possible to identify genes that showed differences in expression levels in response to foliar/culture medium application and genotype. Alaser-capture micro-dissection device allowing for dissection and transcriptome analysis of single cells were used for isolating the different cell types in the metal unloading pathway from the phloem to the endosperm cavity.

Interaction between Partners and WPs

The WP was designed as a well-structured collaboration between six Partners that directly contributed to the current WP. Partner KVL provided the necessary backup with respect to elemental analysis; Partner UBT ensured the availability of different barley genotypes and Partners York, MPS and UZH assisted in the assembly and use of the microarrays. There was also a well-designed strategy for the dissemination of the microarray results into the other WPs operated by partners UBT, MPS, York and South.

Achievements in relation to state-of-the-art

Nutrients destined for the developing cereal grain encounter several restricting barriers on their path towards their final storage site in the grain. We considered the loading of the grain a bottleneck for metal absorption and deposition into the cereal grain. A major objective of the WP was to identify the bottlenecks in barley for the transport of the elements Cd, Fe, Zn, Mn and Se. Such knowledge is essential for future creation of new cultivars with improved heavy metal management.

As a first step towards identifying genes essential for heavy metal handling in barley, the heavy metal-related transcriptome was characterised. Bioinformatic analysis identified more than 100 putative genes involved in transport and deposition of heavy metals in the plant. The genes constitute into 11 families (e.g. ZIP, Nramp, ABC, CDF type, Ptype ATPases and YSL) (**Table 1**).

Gene superfamily	Subfamily	Common name	# in Rice	# identified in barley databases	# present on 22K Barley GeneChip
P-type ATPase	P_{1B}-type ATPase	HMA	9	10	4
Zrt-, Irt-like Protein		ZIP	12	8	8
Cation Diffusion Facilitator (CDF)		MTP	11	12	4
Natural resistance associated macrophage proteins		Nramp	3	4	3
Ca^{2+}-sensitive cross-complementer 1 (CCC1)	Vacuolar iron transporter 1	VIT1	2	2	2
Major facilitator superfamily of membrane proteins	Zinc-Induced Facilitator1	ZIF1	1	1	1
Ca^{2+}/cation antiporter (CaCA)	Cation exchanger	CAX	6	3	3
Oligopeptide transporter family	Yellow Stripe Like	YSL	18	6	5
Metallothionein		MT	9	10	9
Nicotianamine synthase		NAS	3	8	7
Nicotianamine aminotransferase		NAAT	6	4	2
Total			79	68	48

Table 1. Expression of heavy metal related genesin barley.

In order to identify transporters and chelating agents that may be involved in transport and deposition of metals in the barley grain, expression profiles were generated of four different tissue types: the transfer cells, the aleurone layer, the endosperm, and the embryo. Cells from these tissues were isolated with the 'laser capture microdissection' technology and the extracted RNA was subjected to T7-based amplification (**Figure 3**).

The amplified RNA was subsequently hybridized to Affymetrix 22K Barley GeneChips. Likewise, RNA was transcribed into cDNA and subjected to quantitative realtime PCR to verify expression of candidate genes. On the basis of the expression levels of a number of metal homeostasis genes, working models have been proposed for the translocation of Zn and Fe from the phloem to the storage sites in the developing grain (see **Figure 16** below).

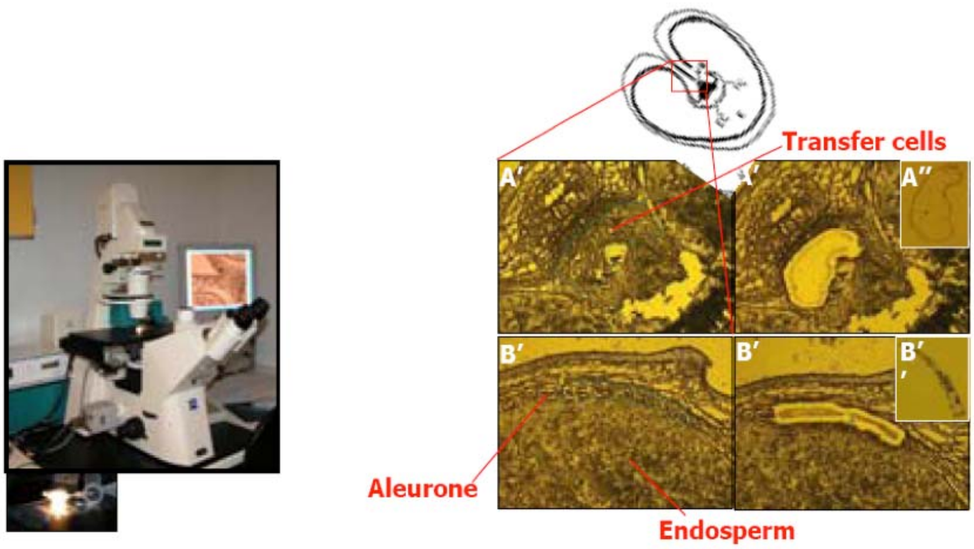

Figure. 3. Laser capture microdissection: Gene discovery and transcriptome analyses from individual cell and tissue types. In order to obtain an expression profile of genes involved in metal transport and deposition in specific tissues of the grain, the laser capture technique was

employed. This technique enabled us to isolate RNA from individual cells from tissue sections. RNA was amplified and reverse transcribed into cDNA and used for microarray hybridizations, real-time PCR analyses and next generation sequencing to establish the expression patterns of particularly relevant genes.

The pathway models described (see also **Figure 16**) are the first attempts to combine knowledge on barley grain structure, grain loading and tissue specific gene expression to build a roadmap for loading minerals into the cereal grain

Conclusions

The transcription analysis and modelling has provided evidence of the function of central proteins important for the grain loading and storage of heavy metals. To get a better understanding of gene function, a range of central genes has been selected for genetic manipulation. The transgenic manipulations are important elements in other work packages of Pillar IV in PHIME. The hypothesis is that up- and downregulation of the involved genes will alter the grain storage location and capacity.

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Workpackage IV:1:3 Assessing natural diversity of heavy metal transport

Summary of WP objectives

Rationale

Natural diversity is significant in most crops. For example, between different wheat cultivars, mean values of grain Fe ranges between 25 and 56 μg $g^{-1}$and Zn concentrations between 25 and 64 μg g^{-1}. By measuring the messenger RNA levels of all expressed genes relative to one another, transcript profiling provides a powerful method for understanding how organisms work under heavy metal stress and how they have adapted their genetic profile towards solving the associated problems.

Main objective of this WP was to establish a foundation for identifying the factors determining micronutrient and toxic metal levels in barley grain. Barley served as a relevant and suitable model system for monocots. Currently, worldwide breeding efforts aiming at improving micronutrient supply (=biofortification) suffer from a lack of knowledge about the molecular basis of the respective traits. As a first step towards our goal we determined the extent of natural variation in grain micronutrient concentrations. Based on these data, (i) in-depth physiological and molecular analyses of extreme genotypes, and (ii) genetic analyses of grain metal loading were pursued.

Contractors involved
UBT (WP leader), UHEI, RUB

Degree to which the objectives were reached
All objectives were reached. Going far beyond the objectives initially proposed, we explored natural diversity in barley grain micronutrient concentrations to a much larger extent. As a consequence, we could also pursue genetic analyses to greater depths than appeared feasible at the outset. This significant expansion of scope and workload inevitably resulted in delays. However, institutional support for the remaining data analyses and publication work is secured.

Methodologies and approaches employed

Experimentation
Essentially three different barley populations were analyzed for variation in grain micronutrient concentrations. First, a PHIME collection of about 140 cultivars was assembled from the Barley Core Collection. Guiding principle was maximization of geographic origins and soil types represented. In addition, the ICARDA collection and introgression lines of the land race *Hordeum spontaneum* into *Hordeum vulgare* cv Scarlett were analyzed by ICP-OES. Plants were grown in multiple field environments and in greenhouses.

Marker data available for the ICARDA collection and the introgression lines were used to identify genomic regions associated with differences in micronutrient levels.

Extreme genotypes were subjected to in-depth analysis. Metal tolerance and metal accumulation were studied at the seedling stage. Grains were dissected and elemental profiles recorded. Grain loading was investigated in a large-scale transcriptome experiment using commercial barley microarrays.

Interaction between Partners and WPs

Experiments were carried out in cooperation between Partners UBT, UHEI and RUB. Results of other WPs provided tools, and were fed into the selection of lines and details of the experimental set-up. Initial screening of barley cultivars was carried out by Partners UBT and UHEI. Tools for the microarray analysis were provided by WP IV:1:2. Design of the detailed analysis were determined based on results of WP IV:1:1. Laboratory data on metal accumulation and tolerance for the selected lines were compared with field data with the help of WP IV:3:4 Partners.

Achievements in relation to state-of-the-art

To our knowledge we conducted the largest screening of natural variation in barley grain micronutrient concentrations to date. Our results demonstrate considerable diversity. We obtained up to 4fold differences between genotypes for the biofortification target micronutrients Zn and Fe. Remarkably, ranges were similar for the two entirely independent collections (Fig. 1) and under a variety of conditions, indicating a large genotype contribution.

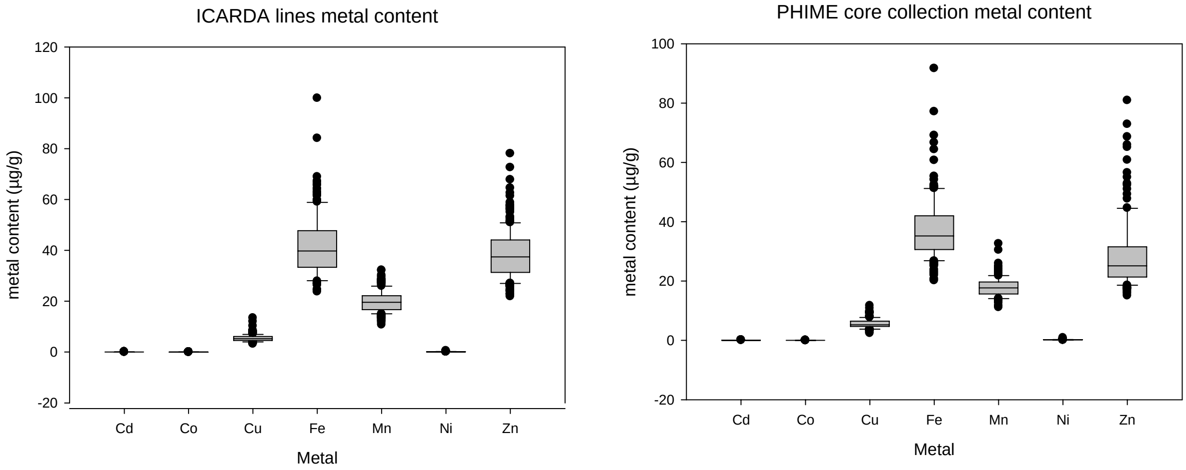

Figure 1 Natural diversity of barley grain micronutrient content. The extent of variation in Zn and Fe concentration is similar for the ICARDA collection (ca. 170 genotypes) and the PHIME collection (ca. 140 genotypes).

Inclusion of more than the initially proposed cultivar collection of about 100 lines was done in order to enable independent confirmation of natural diversity (Fig. 1) and to increase the chances for meaningful genetic analysis by fully exploiting the potential of biological material made available only after the start of the PHIME project, namely the extensively genotyped ICARDA collection and introgression lines of the land race *Hordeum spontaneum* into *Hordeum vulgare* cv Scarlett. Based on our phenotype data and marker information contributed by collaborators it was possible to map QTLs for micronutrient content in both populations. As expected the genetic architecture of mineral content traits is quite complex. Still, our results lay the foundation for the molecular dissection of grain micronutrient natural variation in barley. We are not aware of any similar data.

Identification of extreme genotypes allowed physiological and molecular analysis of metal accumulation in barley grains. Seed dissection, for instance, demonstrated even contribution of embryo, endosperm and husk to differences in accumulation rates.

The transcriptome analysis of extreme cultivars represents to our knowledge the most extensive study on barley micronutrient grain filling conducted so far. These data will also inform many other projects related to grain filling.

Conclusions

Barley as one of the major crops worldwide that, in addition, serves as a genetically less complex model for other monocots including wheat, shows substantial variation in grain micronutrient composition. Because the genotype influence is considerable, this variation means that biofortification targets such as a 3fold increase in Zn and Fe concentrations can be reached.

Our genetic analyses show that the contribution of single loci is small – as is the case for many other agronomically important traits. Major future efforts will be required to dissect the underlying mechanisms. Our results lay a solid foundation for such studies.

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Sub-Pillar IV:2 Characterisation of heavy metal-related genes identified in barley

Background

Crop plants, such as barley, contain their own genes for nutrient metal homeostasis and toxic metaldetoxification, but very little is known about these to date. Multiple homologues of suchArabidopsis genes have been identified in silico in barley and rice. Thus there is a plethora of novelgenes in the crop species for exploitation with great potential for micronutrient accumulation andmetal detoxification.

Plant cells – like all eukaryotic cells – possess an elaborate system of transition metal influx andefflux transporters that ensure transport between cells and cellular compartments. A number ofthese transporters have now been characterised mainly from Arabidopsis thaliana or metalhyperaccumulating species. They belong to the following membrane transport protein families: theZRT-IRT-related (ZIP) proteins, P_{IB}-type heavy metal ATPases (HMA), cation diffusion facilitator (CDF) proteins known as metal tolerance proteins (MTP) in plants, the natural-resistance-associatedmacrophage protein family (NRAMP), the yellow-stripe 1-like (YSL) family, the multidrug effluxtransporter (MATE) family and the ATP-binding-cassette (ABC) transporters. In addition to theirprimary substrate, for example Zn, many of these transporters are also able to transport otheressential micronutrients (e.g., Mn or Fe) or toxic metals (e.g., Cd or Pb). Different primary substratespecificities are commonly found among different members of the same protein family.

In addition to metal transporters, low-molecular-weight metal chelator molecules (e.g., phytochelatins, glutathione and non-protein amino acids such as nicotianamine) are of key importance in controlling free metal ion concentrations as well as the mobility and distribution ofmetals within the plant and within each individual cell. Since some metal transporters use the freemetal ion as substrate, whereas other transporters use metal as metal-chelate complex, metalchelators control the availability of metal species for transport across membranes and thus thedistribution of metals within the plant.

Objectives

i) To characterise further genes that have been identified during the course of this project;

ii) To extend these studies to the protein level in this way offering a detailed characterisation of thegene involved;

iii) To employ protein engineering to alter the properties of selected key transporters for improvedheavy metal homeostasis.

Major results and degree to which the objectives were reached

All objectives were reached. Going far beyond the objectives initially proposed, we explored natural diversity in barley grain micronutrient concentrations to a much larger extent. As a consequence, we could also pursue genetic analyses to greater depths than appeared feasible at the outset. This significant expansion of scope and workload inevitably resulted in delays. However, institutional support for the remaining data analyses and publication work is secured, which implies that we can expect publication in the future of important results obtained from the work in this WP.

WP IV:2:1 Functional characterisation of membrane proteins carrying out heavy metal transport in barley

Summary of WP objectives

The objective of this workpackage was to obtain knowledge of genes/proteins that can be used for molecular breeding approaches designed to increase the concentrations in bioavailable micronutrients, primarily Fe and Zn, and to decrease the accumulation of harmful trace elements, in particular Cd and As, in cereal grains. We specifically targeted genes encoding proteins with potential roles in metal binding or metal chelator biosynthesis and in the transport of metal cations, chelators or chelates across membranes. The knowledge aimed for was (a) the identification of candidate genes/proteins and (b) the detailed characterization of their physiological functions.

Contractors involved

MPS/UHEI/RUB (WP leaders), UBT, FLAKKE, UZH, YORK

Degree to which the objectives were reached

All objectives were reached. Going far beyond the objectives put forward at the outset, several additional genes of major importance in trace element accumulation pathways were identified and functionally characterized in much detail. A number of transgenic barley plants have been generated, which are now available to assess the effects of the introduced transgenes on grain metal contents.

Methodologies and approaches employed

Experimentation
Barley (*Hordeum vulgare*) served as a model cereal crop. To identify genes/proteins of interest, we used an *in silico* approach based on existing knowledge in *A. thaliana* and rice, vacuolar membrane proteomics and cell-type specific expression analyses in the barley grain. As tools to identify the functions of newly identified genes, we used expression in suitable yeast mutants, physiological characterization of Arabidopsis mutants and overexpressing lines and biochemical studies. For functional characterization, we used a large variety of techniques including expression analysis, transcript and protein localization and metal tolerance and metal accumulation assays.

Achievements in relation to the state-of-the-art
Major progress was made in our fundamental understanding of the pathways of trace element movement in plants, in particular concerning the key bottleneck processes of root-to-shoot metal movement and sequestration in cell vacuoles.

- Our work has particularly highlighted the importance of the metal chelators nicotianamine (Fe, Zn, Cd) and phytochelatins (Cd, As, Zn) in these two processes, thereby expanding the known functions for chelators and chelator transport processes at the whole-plant level as well as the range of metals known to be affected by them.
- Work on a metal-hyperaccumulating and -hypertolerant Arabidopsis relative clarified the role for P-type ATPase superfamily proteins in both detoxification and root-to-shoot flux of Zn and Cd, and pinpointed how these processes can be enhanced in plants. This knowledge was then applied in WP IV:3:3.
- An entirely novel family of metal transporters, the PCR transporters, was discovered, and members of it were found to be important for Cd and Zn tolerance as well as root-to-shoot Zn transport.
- In addition, a member of the ABC membrane transporter family was shown to contribute to Cd detoxification.
- Novel cellular pathways of metal movement have been newly identified, in particular the involvement of Golgi vesicles in Mn detoxification through MTP11 and the importance of Mn transit through the vacuole through NRAMP3 and NRAMP4 for growth under Mn deficiency.
- The specific functions of HvIRT1 in Mn uptake and HvMTP1 in Zn storage were elucidated. In addition, vacuole membrane proteomics were established in barley and used for the quantitative investigation of responses to Cd exposure. This approach revealed the localization of a number of barley proteins and pinpointed how knowledge on the metal homeostasis network gained in model plants can be transferred to barley.
- The proteins responsible for the vacuolar sequestration of phytochelatins and their complexes, a process known biochemically for almost 20 years, have finally been molecularly identified in PHIME and shown to have particular relevance for the movement of As within the plant.

Conclusions

Numerous key players in the metal homeostasis network have been identified and functionally characterized. This leads to a substantially improved fundamental molecular-physiological understanding of the pathways of metal and metalloid movement and accumulation in plants. Our novel results have generated newly arising scientific questions requiring further research. Results obtained in model species can generally be transferred to barley as an example of a cereal crop. However, this is still technically difficult and time-consuming, and additional aspects need to be considered in this crop plant.

This workpackage has generated a suite of novel tools and new knowledge of how to use them in approaches aimed at the biofortification of crops and at minimizing the accumulation of harmful trace elements. A few of the newly identified and characterized genes have already been introduced into crop plants for proof-of-concept within PHIME (see workpackage IV:3.3).

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WP IV:2.2 Identification of the specificity determinants of metal homeostasisproteins and modification of metal specificities

Summary of WP objectives

- To identify new metal selectivity determinants in metal transporters and homeostasis proteins.
- To modify these specificities in order to improve cellular nutrient metal enrichment and storage and to optimise cellular toxic metal exclusion or efflux.

Contractors involved

York/JIC (WP leaders), South, UBT, MPS, KVL.

Degree to which the objectives were reached

All objectives were achieved.

Methodologies and approaches employed

Rationale

It is well established that key metal homeostasis proteins have limited metal specificity. Many heavy metals enter plants roots via non-specific channel proteins. The P_{IB}-type metal pump HMA4is also lacking specificity and is critical for root-to-shoot translocation of the essential micronutrientZn as well as the toxic heavy metal Cd. Understanding the determinants of metal specificity of MTP-related CDF proteins will open possibilities for the generation of metal sinks through sequestration of metals in the vacuole of specific tissues. Also, the synthesis of ligands determines metal partitioning between roots and above-ground parts. Responsible enzymes such as phytochelatin synthases are known to be differentially activated by metal ions. The vacuole is known to be the main store for heavy metals. Efficient transport and storage of heavy metals in the vacuole will result in a decreased mobilisation during the grain filling period. It was therefore an important task to identify vacuolar heavy metal transporters.

Experimentation

Characterised metal homeostasis genes have been used, ion binding sites characterised and specificity introduced by established in vitro recombination and mutation techniques. Structural determinants of ion specificity have been determined in detail. We attempted to increase specificities of P_{IB}-type ATPases and CDFproteins for Zn thus lowering the amount of Cd transported. We attempted to increase Zn^{2+} activation of PC synthases and lower Cd^{2+} activation. This was achieved via targeted site-directed mutagenesis and random mutagenesis using transgenic yeast as a genetic system for isolation of mutant transporters with altered specificity. Unicellular model systems were used for screening and fast characterisation in order to identify determinants of metal specificity and activity, as well as to optimise specificity. These techniques were used aiming to identify determinants of specificity and efficiency of cellular nutrient metal enrichment or storage, or of cellular toxic metal exclusion or efflux.

Interaction between Partners and WPs

The plan was to obtain candidate genes in metal transport and chelator biosynthesis from the WPs of SP IV:1. The work in this WP was performed in close collaboration between a number of Partners. Depending on their protein family of expertise, different partners performed the molecular and functional characterisation of members of different protein families, namely Partners South, KVL and Flakke will focus on P1B-metal ATPases/pumps, Partner UBT focused on nicotianamine synthase isoforms, and PartnersYork and South focussed focus on CDF family metal transporters. The proteomics and the characterisation of ABC family transporter proteins was performed by Partner UZH. After initial characterisation, a subset of the cloned cDNAs was transferred into WP IV:2:2 for optimisation of transporter properties. A subset of these and of the remainder of cDNAs was additionally transferred into appropriate WPs of SP IV:3, dependingon the function of each of the proteins established in this WP.There was additional joint coordination for all WPs within WP IV to ensure efficient coordinationof the work and to ensure the exchange of information. Cross-cutting WP C:9 was involved for dissemination.

Achievements in relation to the state-of-the-art

The Cation Diffusion Facilitator (CDF) family is a ubiquitous family of heavy metal transporters. Much interest in this family has focused on implications for human health and bioremediation. In this work a broad phylogenetic study has been undertaken which, considered in the context of the functional characteristics of some fully characterised CDF transporters, has aimed at identifying molecular determinants of substrate selectivity and at suggesting metal specificity for newly identified CDF transporters.

Representative CDF members from all three kingdoms of life (Archaea, Eubacteria, Eukaryotes) were retrieved from genomic databases. Protein sequence alignment has allowed detection of a modified signature that can be used to identify new hypothetical CDF members. Phylogenetic reconstruction has classified the majority of CDF family members into three groups, each containing characterised members that share the same specificity towards the principally-transported metal, i.e. Zn, Fe/Zn or Mn. The metal selectivity of newly identified CDF transporters could be inferred by their position in one of these groups. The function of some conserved amino acids was assessed by site-directed mutagenesis in the poplar Zn^{2+} transporter *PtdMTP1* and compared with similar experiments performed in prokaryotic members. An essential structural role can be assigned to a widely conserved glycine residue, while aspartate and histidine residues, highly conserved in putative transmembrane domains, might be involved in metal transport. A potential role of group-conserved amino acid residues in metal specificity is evident.

The phylogenetic and functional analyses have allowed the identification of three major substrate-specific CDF groups. The metal selectivity of newly identified CDF transporters can be inferred by their position in one of these groups. The modified signature sequence proposed as a result of this WP can thus be used to identify new hypothetical CDF members.

Manganese toxicity is a major problem for plant growth in acidic soils, but cellular mechanisms that facilitate growth in such conditions have not been clearly delineated. Established mechanisms that counter metal toxicity in plants involve chelation and

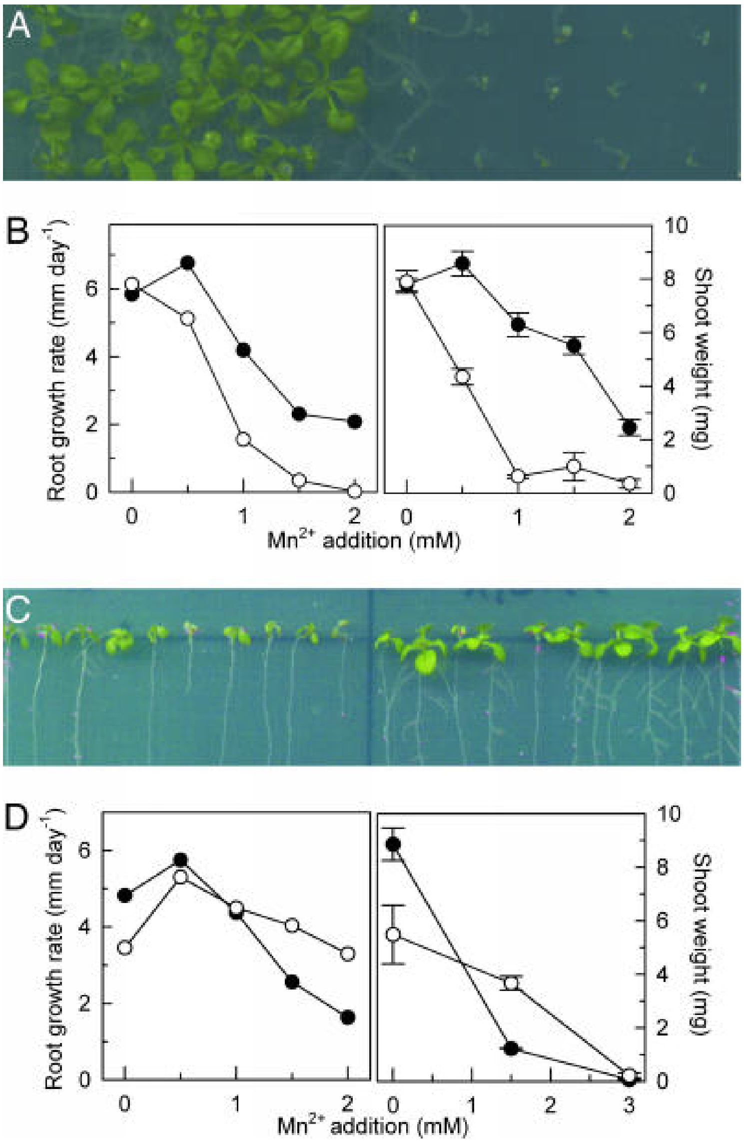

Figure 5. The Arabidopsis mtp11-1 mutant is hypersensitive to Mn, whereas an MTP11 overexpressor is Mn-hypertolerant. (A) Growth of wild-type (Left) and mtp11-1 (Right) seedlings on a half-strength MS plate supplemented with 1 mM MnSO4. (B Left) Effect of Mn on root elongation of wild type (filled circles) and mtp11-1 mutant (open circles). Each data point is a linear regression of root elongation of 30 seedlings measured at 4, 6, 8, 11, and 13 days after germination. Regression coefficients of all points are >0.95. (B Right) Effect of Mn on shoot fresh weight of 17-day-old wild-type (filled circles) and mtp11-1 mutant (open circles) seedlings grown on horizontal plates. Data are the means ± SEM of three plates (25 seedlings per line and plate). (C) Growth of Col 0 (Left) and 35S::AtMTP11 line 8 (Right) on a near-vertical half-strength MS plate supplemented with 1.5 mM MnSO4. (D Left) Effect of Mn on root elongation of wild type (filled circles) and 35S::AtMTP11 line 3 (open circles). Experimental details are as in B. (D Right) Effect of Mn on shoot fresh weight of 13-day-old wild type (filled circles) and 35S::AtMTP11 line 3 (open circles) grown on near-vertical plates. Data are the means ± SEM of three plates (10 seedlings per line and plate). For A–D, experiments were repeated twice with similar results.

AtMTP11

cytoplasmic export of the metal across the plasma or vacuolar membranes out of the cell or sequestered into a large organelle, respectively. We found that expression of the Arabidopsis and poplar MTP11 cation diffusion facilitators in a manganese-hypersensitive yeast mutant restores manganese tolerance to wild-type levels. Microsomes from yeast expressing exhibited enhanced manganese uptake. In accord with a presumed function of MTP11 in manganese tolerance, Arabidopsis mtp11 mutants were hypersensitive to elevated levels of manganese, whereas plants overexpressing MTP11 are hypertolerant. In contrast, sensitivity to manganese deficiency was slightly decreased in mutants and increased in overexpressing lines. Promoter-GUS studies showed that AtMTP11 is most highly expressed in root tips, shoot margins, and hydathodes, but not in epidermal cells and trichomes, which are generally associated with manganese accumulation. Surprisingly, imaging of MTP11-EYFP fusions demonstrated that MTP11 localizes neither to the plasma membrane nor to the vacuole, but to a punctate endomembrane compartment that largely coincides with the distribution of the trans-Golgi marker sialyl transferase. Golgi-based manganese accumulation might therefore result in manganese tolerance through vesicular trafficking and exocytosis. In accord with this proposal, Arabidopsis mtp11 mutants exhibit enhanced manganese concentrations in shoots and roots. Consequently, we have propose that Golgi-mediated exocytosis comprises a conserved mechanism for heavy metal tolerance in plants.

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Sub-Pillar IV:3 Modification of crop plants towards toxicologically enhanced varieties

Background

In this Sub-Pillar it was intended to use the knowledge obtained through the Project for altering heavy metal contents of the edible parts of crop plants. Focus was on changing the toxicological status of the barley grain. However, tobacco and tomato, which serve as model plants for dicotylodoneus leaves and fruits, respectively, were also modified.

The focus was initially on P_{IB}-ATPases, a family of potentially important heavy-metal pumps. This gene family has been chosen in particular because the P_{IB}-type ATPases (also known as the heavy metal ATPases/ HMAs), are emerging as central players in transition metal transport in plants. Once heavy metals have entered plant roots, these pumps are essential for their subsequent transport via the vascular system to aerial parts. At later stages of development, they are hypothesised to be essential for the direct pumping of metals into the grains of cereals. A natural substrate for a subset of P_{IB}-ATPases is Zn. Mutant studies have demonstrated that these are essential for Zn nutrition. However, as they poorly distinguish between Zn and Cd, the very same pumps are likely to be responsible for Cd deposition in edible plant parts.

Recent evidence suggests that P_{1B}-ATPases have a variety of physiological functions in addition to the translocation of Zn, namely in detoxification processes at elevated Cd and Zn concentrations. Their importance in transition metal transport (Zn, Cd and Pb) and homeostasis (Zn) in the model plant Arabidopsis has now been clearly demonstrated and evidence suggests that they have a key role in the mechanisms underlying hyperaccumulation of metals in particular species.

Understanding the role of P_{1B}-ATPases in metal uptake, distribution and homeostasis in crops is essential for developing strategies to modulate the metal content of grain, which will have a direct impact on metals entering the human food chain. Genes involved in transport of heavy metals to the vacuole, a compartment for heavy metal detoxification, were also targeted.

Subsequently, the expression of the selected genes were manipulated to investigate the physiological effects and role in heavy metal homeostasis. The tools were overexpression, RNA interference and TILLING facilities that are currently becoming available. This will allow for a detailed study of their effect on uptake, sequestration and root-to-shoot translocation of the metals Fe, Mn, Se, Zn, Hg as well as Cd, paying particular attention to their potential role in the transfer of metals into the aleurone and endosperm of the barley grain. This has provided the first step towards creating new cultivars with improved content of heavy metals.

Objectives

i) To engineer cereals with increased Zn content, particularly in the barley grain, but at the same time avoiding concomitant accumulation of toxic Cd ions, which are also transported by these proteins;

ii) To use P_{1B}-ATPases as subjects for a focused case study on the introduction of heavy metals into the food chain;

iii) To test the hypothesis that P_{1B}-type ATPases could be modified to alter profiles of micronutrient nutrition (in particular Zn) and heavy metal detoxification (in particular Cd) in barley;

iv) To test the potential of genes selected in previous WPs by generating transformed dicotyledonous plant crops with increased content of Zn and reduced concentration of Cd.

WP IV:3.1 A case study: Characterisation of the complement of P_{1B}-ATPase Zn and Cd pumps

Summary description of WP objectives

i) To examine the contribution of P_{1B}-type ATPases to micronutrient nutrition (in particular Zn) and heavy metal detoxification (in particular Cd);

ii) To determine the contribution of P_{1B}-type ATPases to delivering metals to the grain;

iii) To investigate whether altering the expression levels of P_{1B}-type ATPases can influence heavy metal transport, accumulation or detoxification.

Contractors involved
South (WP leader), KVL, Flakke.

Degree to which the objectives were reached
All objectives were achieved.

Methodologies and approaches employed

In this WP, the contribution of a selected gene family to heavy metal handling by a crop plant has been determined. The focus has specifically been on P_{1B}-type ATPases, a family of potentially important heavy-metal pumps. In particular, the contribution of P_{1B}-type ATPases to delivering metals to the grain and also their potential for detoxification of harmful metals were determined. For this work the Partners have i) determined the exact *in planta* localisation of barley P_{1B}-ATPases; ii) altered their expression levels *in situ*, in order to study the physiological effects on heavy metal handling in barley.

The barley P_{1B}-ATPase genes including promoter regions were cloned. The promoter regions were used as a basis for promoter reporter gene analysis. Constructs were made in which cDNAs for barley P_{1B}-ATPases fused at the gene level to GUS and GFP are under control of these promoters. The ATPase proteins were localised *in planta* at the cellular and subcellular level, employing GUS reporter gene analysis and GFP marker fusion technology and immunological approaches. Further, the role of P_{1B}-ATPases in Zn nutrition and Cd detoxification were determined, employing transgenic plants with altered expression of these pumps. The expression levels of P_{1B}-ATPases were manipulated in barley and the physiological effects investigated, particularly with respect to Zn and Cd homeostasis. This was done by expressing Arabidopsis and barley P_{1B}-ATPases in barley. Subsequently, RNAi techniques were used to down-regulate expression, using the crop genes obtained in the project. The performance of the plants was investigated by assessing the growth and development of the transformed plants when grown on a range of metals and also assessing whether the metal concentration is altered in different tissues. A particular focus was the delivery of metals to the barley grain.

Interaction between Partners and WPs

The research activities of several partners were closely coordinated: Partners SouthU and KVL have cloned the barley genes and prepared the GUS, GFP and RNAi constructs. These were supplied to Partner Flakke for barley transformation. The resulting transgenic barley was analysed by Partners SouthU and KVL. There were coordination with: WP IV:1.2, where transcriptome information was used in the further analysis of P1B-ATPases; in WP IV:2.1, where P1B-ATPases were functionally characterised by expression in unicellular model expression systems; in WP IV:2.2, where structural determinants of ion specificity were determined and P1B-ATPases were engineered towards altered specificity; in WP IV:3.2, where P1B-ATPases were tested for improving neutralisation of heavy metals in barley vacuoles; in WP IV:3.3, where dicotyledonouscrop plants were transformed with P1B-ATPases; in WP IV:3.4, where transgenic plants modified in P1B-ATPase expression were compared under glasshouse conditions to those transformed with other metal transporters/enzymes. Several cross-cutting WPs have been engaged and WP C:9 helped with dissemination of information.

Achievements in relation to state-of-the-art

We identified nine P_{1B}-ATPases in barley from EST analysis and from comparisons with Arabidopsis, three pumps were predicted to have a role in Zn/Cd transport, HvHMA1, 2 and 3. We focused mainly on HvHMA 1 and 2 in this WP, and provided evidence for their role in heavy-metal transport. To show that they could function in Zn nutrition or heavy metal (Cd) detoxification, it was necessary to show that they could transport these metals. cDNAs were cloned for both, and structure/function analysis was performed by heterologously expressing these pumps in yeast and carrying out metal-sensitivity/resistance tests. Results indicate that both pumps have motifs and key residues characteristic of P_{1B}-ATPases; HvHMA1 can transport Zn, but also Cu, Co, Cd, Ca, Mn and Fe in yeast, while HvHMA2 transports Zn and Cd. Transport rather than binding is suggested by the fact that mutating a key residue critical for transport function in P-type ATPases abolished the particular metal-dependent sensitivity/resistance.

We also investigated the *in planta* functioning of these pumps by expressing them in Arabidopsis mutants that are defective in particular P_{1B}-ATPases. The Arabidopsis *hma2/hma4* mutant is severely stunted, due to the lack of Zn translocation from roots to shoots. HvHMA2 restored the wild-type phenotype in this mutant indicating that HvHMA2 may have functional equivalence to AtHMA2 and AtHMA4 in Zn transport in the plant. HvHMA1 was expressed in the Arabidopsis *hma1* mutant and showed that it partially rescued the high light phenotype (which has been suggested as being due to Cu transport) and also it rescued the Zn-sensitive phenotype of this mutant. This suggests that AtHMA1 can function in Cu and Zn transport *in planta*.

In order to understand their contribution to delivering metals to the grain, we investigated their expression within the plant. AtHMA1 was expressed at highest levels in leaves and grains and was localised to chloroplasts and intracellular compartments of aleurone layer cells. HvHMA1 expression was induced by Zn deficiency, whereas toxic levels of Zn and other metals inhibited transcription. HvHMA2 is expressed throughout the plant and within the grain it was shown to have highest expression levels in the transfer cells suggesting a role in pumping Zn into the endosperm cavity to supply the filial tissues. At the cellular level, HvHMA1 was localized to chloroplasts and intracellular compartments of aleurone layer cells. HvHMA2 appeared to localise to plasma membrane when expressed in Arabidopsis, but its localisation in barley has not yet been determined.

To investigate whether altering the expression levels of P_{1B}-type ATPases can influence heavy metal transport, accumulation or detoxification in barley we used RNA interference to alter expression levels. Only plants with reduced HvHMA1 have been analysed. These showed no change in Zn content of leaves compared to wild type, but had a significant increase of Zn in grains. We have produced barley plants that are over-expressing *HvHMA2;* however, these plants still remain to be characterized in detail.

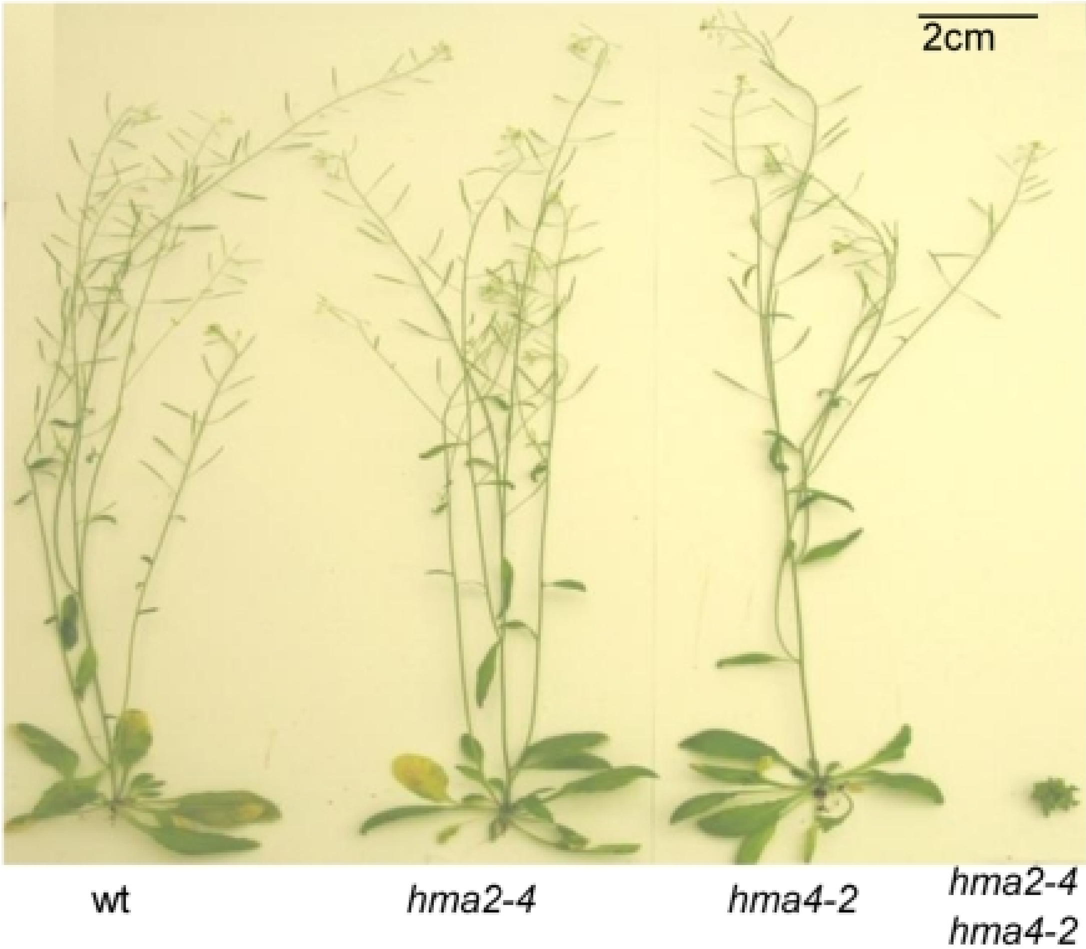

Figure 7. Comparison of wild-type and mutant plants grown on soil. Plants were grown for 42 days on soil under identical conditions in a controlled-environment growth room (22°C 16 h light, 20°C 8 h dark cycle). Arabidopsis thaliana (Columbia) wt, hma2-4 (SALK_034393), hma4-2 (SALK_050924) and the double hma2-4 hma4-2 mutant are shown.

In relation to examining the contribution of P_{1B}-type ATPases to micronutrient nutrition, it was also found that isolated barley chloroplasts exported Zn when supplied with Mg-ATP and this transport was inhibited by thapsigargin (an inhibitor that has previously been shown to inhibit AtHMA1). Based on this, and the above, it appears that HvHMA1 is a broad-specificity metal exporter from chloroplasts that might serve as a scavenging mechanism for mobilizing plastid Zn during cellular Zn deficiency. Furthermore, HvHMA1 might be involved in mobilizing Zn from grain intracellular compartments in aleurone layer cells during grain filling and germination. From the range of information obtained for HvHMA2, we suggest it is a plasma membrane transporter that functions in Zn/Cd export and has a role in root to shoot transport of Zn (and Cd when present). It also may have a role in exporting Zn from maternal transfer cells to the endosperm cavity for supplying the grain during development.

Methodologies and approaches employed

RT-PCR and real-time PCR. Reporter gene analysis. RNA interference. Heterologous expression in yeast. Yeast metal sensitivity/resistance tests. Ectopic expression. ICP-MS.

Achievements in relation to the state-of-the-art

The ultimate goal of modern agriculture is to produce nutritious and safe foods in sufficient quantities and in a sustainable manner. Manipulation of crops to improve their nutritional value (biofortification) and optimization of plants for removal of toxic metals from contaminated soils (phytoremediation) are major goals. Further understanding of membrane transporters with roles in Zn and Cd transport would be useful for both aspects. Taken together, our results have contributed to the understanding of the role of P_{1B}-ATPases in barley, an important crop plant.

Conclusions

HvHMA1 and HvHMA2 are heavy-metal pumps found in barley. It appears that HvHMA1 is a broad-specificity metal exporter from chloroplasts that might serve as a scavenging mechanism for mobilizing plastid Zn during cellular Zn deficiency. Furthermore, HvHMA1 might be involved in mobilizing Zn from grain intracellular compartments in aleurone layer cells during grain filling and germination. From the range of information obtained for HvHMA2, we suggest it is a plasma membrane transporter that functions in Zn/Cd export and has a role in root to shoot transport of Zn (and Cd when present). It also may have a role in exporting Zn from maternal transfer cells to the endosperm cavity for supplying the grain during development.

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Workpackage IV:3:2 Improving neutralization of heavy metals in barley vacuoles

Summary of WP objectives

The objective of this work package has been to capitalise on our knowledge of genes to have a role in transport into barley vacuoles. This will strengthen a sink for heavy-metal immobilisation in vegetative parts of the plant not intended for food consumption. The objective was to introduce some of these candidate genes into model crop plants and to characterise the resulting plants.

Contractors involved
UZH (WP leader), South, UBT, MPS, KVL.

Degree to which the objectives were reached
All objectives were reached.

Methodologies and approaches employed

Experimentation

A few known metal homeostasis proteins are well characterised and are essential for immobilisation and detoxification of heavy metals that have entered the plant body. One known example is the *Arabidopsis thaliana* ZAT or MTP1 protein, which is known to be rather specific in transporting Zn into the vacuole of plant cells. The corresponding gene is well-suited for characterisation upon expression in model crop plants, with the objective to specifically enhance Zn accumulation in target organs by generating an artificial Zn sink. Cell-type specific promoters have been used where appropriate. The introduction of such genes and/or of RNA interference constructs into model crop plants will allow a careful evaluation of their potential to enhance the accumulation of nutrient metals or mediate detoxification or exclusion of harmful metals. This will also generate mechanistic insight into nutrient metal accumulation in the chosen model crops.

Important interaction between Partners and WPs

There has been a close co-operation between Partners and with other WPs. Within WP IV:3.2, the Partners have exchanged their vectors and promoters for cell-type specific expression. Plants were transformed, with several transporters identified in the different groups participating in WP IV:3.2, in order to increase the vacuolar sink for heavy metals. There were also interaction with other WPs: Results have been used from WP VI:1.2, WP IV:1.3 and WP VI:2.1. Results have been provided to WP IV:3.1 (Cereal expression vectors containing cell-type-specific promoter) and WP IV:3.3 (Constructs for cell-type-specific heterologous expression of MTP in barley).

Achievements in relation to the state-of -the-art

In plants the vacuolar functions are the cellular storage of soluble carbohydrates, organic acids, inorganic ions and toxic compounds. Transporters and channels located in the vacuolar membrane, the tonoplast, are modulated by PTMs to facilitate the optimal functioning of a large number of metabolic pathways. As an important achievement, we developed a phosphoproteomic approach for the identification of *in vivo* phosphorylation sites of tonoplast (vacuolar membrane) proteins (Endler et al. 2009).

In this approach, highly purified tonoplast and tonoplast-enriched microsomes were isolated from photosynthetically induced barley (*Hordeum vulgare*) mesophyll protoplasts. Phosphopeptides were enriched and subsequently analysed using LC-ESI-MS/MS. In total, 65 phosphopeptides of 27 known vacuolar membrane proteins were identified, including the two vacuolar proton pumps, aquaporins, CAX transporters, Na^+/H^+ antiporters, as well as other known vacuolar transporters mediating the transfer of potassium, sugars, sulphate and malate. This work has provided a novel source to analyse the regulation of tonoplast proteins by protein phosphorylations, with most phosphorylation sites being highly conserved between *Hordeum vulgare* and *A. thaliana*.

Although the vacuole is the most important final store for toxic heavy metals like Cd, our knowledge on how they are transported into the vacuole is still insufficient. It has been suggested that Cd could be transported as phytochelatin-Cd by an unknown ABC transporter, or in exchange with protons by cation/proton exchanger (CAX) transporters. To unravel the contribution of vacuolar transporters to Cd detoxification, we performed a quantitative proteomics approach (Schneider et al. 2009). Highly purified vacuoles were isolated from barley plants grown under minus, low (20 µM), and high (200 µM) Cd conditions and protein levels of the obtained tonoplast samples were analyzed using isobaric tag for relative and absolute quantitation (iTRAQ). Although 56 vacuolar transporter proteins were identified, only a few were differentially expressed.

Interestingly, the protein ratio of a CAX1a and a natural resistance-associated macrophage protein (NRAMP), responsible for vacuolar Fe export was increased differentially in response to Cd. CAX1a might play a role in vacuolar Cd transport. An increase in NRAMP activity leads to a higher cytosolic Fe concentration, which may prevent the exchange of Fe by toxic Cd. Additionally, an ABC transporter homolog to AtMRP3 showed up-regulation. Under high Cd conditions, the plant response was more specific. Only a protein homologous to AtMRP3 that showed already a response under low Cd conditions, was up-regulated. Interestingly, AtMRP3 is able to partially rescue a Cd-sensitive yeast mutant. The identified transporters are good candidates for Cd-detoxification proteins.

Phytochelatins mediate tolerance to heavy metals in plants and some fungi by sequestering phytochelatin-metal complexes into vacuoles. To date, only *Schizosaccharomyces pombe* Hmt1 has been described as a phytochelatin transporter and attempts to identify orthologous phytochelatin transporters in plants and other organisms have failed. Work in the WP demonstrated that deletion of all vacuolar ABC transporters abolishes phytochelatin accumulation in *S. pombe* vacuoles and abrogates $^{35}S\text{-}PC_2$ uptake into *S. pombe* microsomal vesicles (Mendoza-Cózatl et al. 2010). Systematic analysis of the entire *S. pombe* ABC transporter family identified Abc2 as a full-size ABC transporter (ABCC-type) that mediates phytochelatin transport into vacuoles. The *S. pombeabc1 abc2 abc3 abc4 hmt1* quintuple and *abc2 hmt1* double mutant show no detectable phytochelatins in vacuoles. Abc2 expression restored phytochelatin accumulation into vacuoles and suppressed the Cd sensitivity of the *abc* quintuple mutant.

A novel, unexpected, function of Hmt1 in GS-conjugate transport was also shown. In contrast to Hmt1, Abc2 orthologs are widely distributed among Kingdoms and are proposed as the long-sought vacuolar phytochelatin transporters in plants and other organisms. The presence of these transporters now remains to be demonstrated in barley.

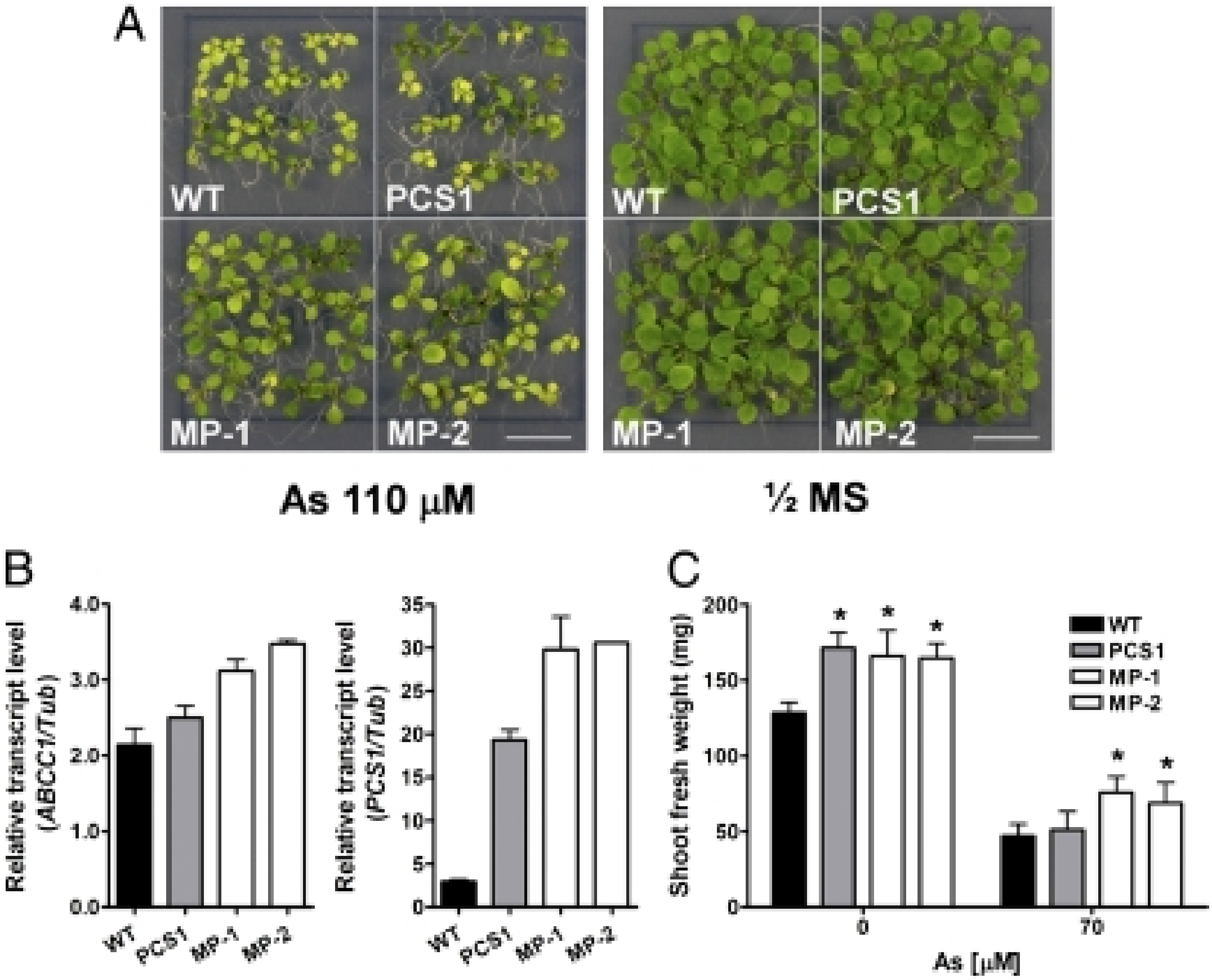

Figure 8. Co-overexpression of PC synthase (AtPCS1) and AtABCC1 results in increased arsenic tolerance. (A) Wild-type plants, plants of a representative AtPCS1-overexpressing line (PCS1), and plants overexpressing AtPCS1 and AtABCC1 (MP-1 and MP-2) grown either on 110 µM As(V)-containing or control half-strength MS plates. (B) Transcript levels of AtABCC1 and AtPCS1 in the plants used in A, determined by qPCR. (C) Shoot fresh weight of wild-type and transgenic plants grown under control conditions or in the presence of 70 µM As(V). Mean ± SEM (from two independent experiments with four replicates each). *P < 0.02 by Student's t test.

Arsenic (As) is an extremely toxic metalloid, causing serious health problems. In Southeast Asia, aquifers providing drinking and agricultural water for tens of millions of people are contaminated with As (WP I:4). To reduce nutritional As intake through the consumption of contaminated plants, identification of the mechanisms for As accumulation and detoxification in plants is a prerequisite.

Phytochelatins (PCs) are glutathione-derived peptides that chelate heavy metals and metalloids such as As, thereby functioning as the first step in their detoxification. Plant vacuoles act as final detoxification stores for heavy metals and As. The essential PC-metal(loid) transporters that sequester toxic metal(loid)s in plant vacuoles have long been sought, but remain unidentified in plants. We managed to show that in the absence of two ABCC-type transporters, AtABCC1 and AtABCC2, *A. thaliana* is extremely sensitive to As and As-based herbicides (Song et al. 2010). Heterologous expression of these ABCC transporters in phytochelatin-producing *S. cerevisiae* enhanced As tolerance and accumulation.

Furthermore, membrane vesicles isolated from these yeasts exhibited a pronounced arsenite [As(III)]-PC(2) transport activity. Vacuoles isolated from *atabcc1 atabcc2* double knockout plants exhibited a very low residual As(III)-PC(2) transport activity, and interestingly, less PC was produced in mutant plants when exposed to As. Overexpression of AtPCS1 and AtABCC1 resulted in plants exhibiting increased As tolerance. Our findings demonstrate that AtABCC1 and AtABCC2 are the long-sought and major vacuolar PC transporters. In the future, mulation of vacuolar PC transporters in other plants, such as barley, may allow engineering of plants suited either for phytoremediation or reduced accumulation of As in edible organs.

Conclusions

The result of the WP has been an important mapping of transport proteins in barley vacuoles relevant for metal transport. Work in other organisms has for the first time characterized Cd and As transporters in the vacuolar membrane. This work has paved the ground for the identification of analogous proteins in the barley vacuolar membrane, a prerequisite for the future manipulation of deposition and storage of harmful metals and metalloids in the vacuole.

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WP IV:3:3 Engineering of dicotyledonous crop plants with improved heavy metal homeostasis

Summary description of WP objectives

Low Zn crops contribute to Zn-deficiency-based malnutrition. Engineering of increased Zn content in plants is one of the aim of ongoing biofortification research. In order to know whether identified genes could be widely used in the engineering of nutritionally improved healthy food, it is essential to find out whether their overexpression in dicotyledonous crop plants results in altered Zn and Cd distribution.

There were two major objectives of the WP IV:3:3:

(i) To generate transformed dicotyledonous crops with increased content of micronutrients (primarily Zn), and reduced concentration of toxic heavy-metal Cd;

(ii) To identify genes, which could be recommended for a wide use in the engineering of nutritionally improved healthy food.

The objectives were reached by introducing candidate genes into model crop dicotyledonous plants and to test whether in the obtained transformants the root-to-shoot Zn translocation barrier was overcome. Moreover, the important part of the study was to learn if the accumulation of toxic Cd was reduced. This will broaden the knowledge on the nutrient and toxic metal accumulation mechanisms, and show the possibility of using them widely to engineer a range of crop species with optimised contents of toxic elements and nutrients.

Contractors involved

WarsU (WP leader), South, UBT, MPS.

Degree to which the objectives were reached

The studies were performed according to the schedule. All objectives were reached. The usefulness of selected genes for the enhancement of Zn concentrations in the shoots was investigated by their heterologous expression. Moreover, we searched for the possibility to reduce the uptake/accumulation of Cd in plants, especially in their transfer to the shoots.

Nine genes chosen for analysis were used for transformation of tobacco, and four genes were expressed in tomato.

Methodologies and approaches employed

Rationale

In order to know whether identified genes could be widely used in the engineering of nutritionally improved healthy food, it is essential to find out whether their overexpression in dicotyledonous crop plants results in altered Zn and Cd distribution. The objective was reached by introducing candidate genes into model crop dicotyledonous plants and to characterise the obtained plants. This will broaden the knowledge of the nutrient and toxic metal accumulation mechanisms and show the possibility of using them widely to engineer a range of crop species with optimised contents of toxic elements and nutrients.

Experimentation

Candidate genes have been overexpressed in tobacco (a model plant for accumulation of metals inleaves) and tomato (a model crop plant for accumulation of metals in edible fruits). Transformation protocols with these species are already available. Genes involved both in the chelation of metals (AhNAS), as well as in transport – P_{1B}-ATPases (HMA) and ABC transporters (MRP) – have been used. It has been of key importance to investigate the change in the metal distribution pattern also in dicotyledonous plants transformed with a native P_{1B}-ATPase clone, as well as mutated versions with high specificity towards Zn and low specificity towards Cd (prepared in WP IV:2:2).Transformed independent lines were characterised with respect to their growth and development responses primarily to Zn and Cd. Metal loading into fruits as well as accumulation pattern in different plant parts from roots to shoots have been identified.

To conclude on the suitability of chosen genes for engineering of enhanced Zn shoot level, it is advisable to express them against different genetic background - in different host plant species. We have chosen tobacco (a model plant for accumulation of metals in shoots, which is easy to transform and regenerate), and tomato (model crop species for accumulation of metals in shoots and fruits, which needs, however, a long-term procedure to get transformants). Due to time-constrains, it was not possible to express all chosen genes in two species.

All genes were cloned by other Partners of Pillar IV from the following species: *Arabidopsis thaliana*; Zn-hyperaccumulator *Arabidopsis halleri*, and from *Hordeum vulgare*. They were expressed either under strong CaMV constitutive promoter or under strong native promoter from *A. halleri*.

The genes selected for the transformation are involved in: (i) chelation and long-distance transport of metals, mainly Fe and Zn (*AhNAS*); (ii) in the control of Zn root-to-shoot translocation either by loading of xylem vessels with metals (transporters P_{1B}-ATPases *HMAs*such as *AhHMA4*; *AtHMA4* and its two mutants; *HvHMA2*), or by releasing of citrate to xylem vessels (*AhFRD3*); (iii) in intracellular store of metals ABCC transporter *AtMRP7* and P_{2A}-ATPase *AtECA3*. All together nine genes were introduced to tobacco and four genes to tomato. Transformed plants were grown in hydroponics, and characterized with respect to their growth and development responses, primarily to: (i) Zn and Cd, metal partitioning, (ii) subcellular localization of proteins, (iii) expression pattern (of transgenes - expecially those under their native promoters, and endogenes of the transgenic plants).

Seeds of generated transgenics were sent to Partner IETU for cultivation on soil with different Zn levels, to determine Zn and Cd concentrations in plant parts - mainly in fruits.

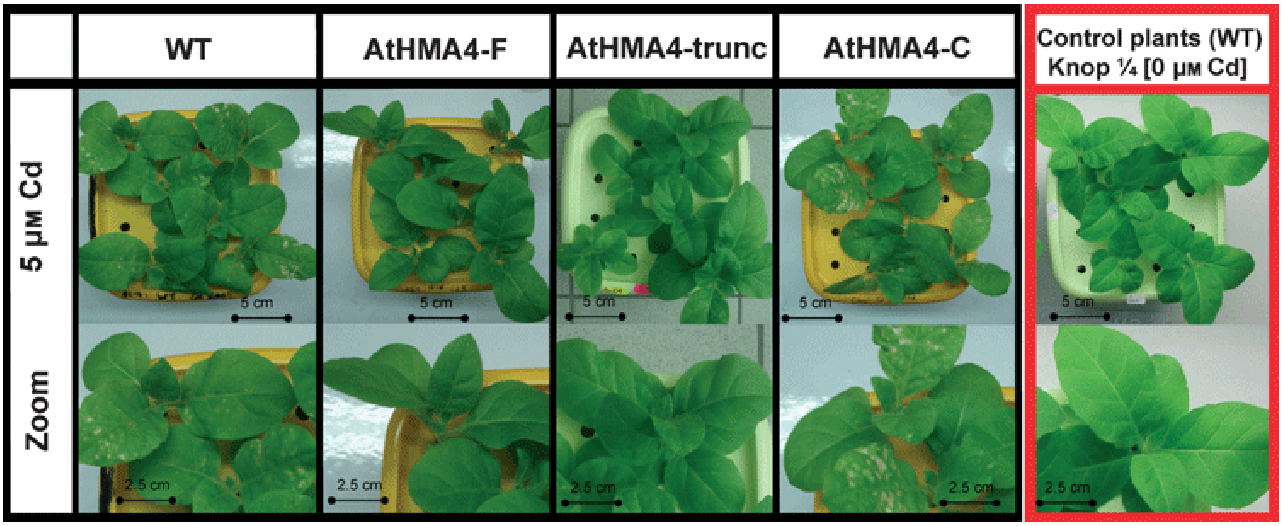

Figure 9. Appearance of 6-week-old AtHMA4-F plants, AtHMA4-trunc plants, AtHMA4-C plants, and the wild type (WT) exposed to 5 µm Cd. Black frame—plants exposed for 8 days to 5 µm Cd. Red frame—WT grown in control medium. Dry matter yield of plant lines grown under control conditions (quarter-strength Knop's solution) did not differ significantly among each other, the same applies to plants grown in the presence of 5 µm Cd. Dry matter of the plants at the end of exposure to 5 µm Cd in grams, Roots: WT 0.0262 ± 0.0092; AtHMA4-F (line 5) 0.0380 ± 0.0034; AtHMA4-trunc (line 9) 0.0320 ± 0.0192; AtHMA4-C (line 16) 0.0229 ± 0.0089; Shoots: WT 0.1275 ± 0.0458; AtHMA4-F (line 5) 0.1498 ± 0.0571; AtHMA4-trunc (line 9) 0.1713 ± 0.0908; AtHMA4-C (line 16) 0.1200 ± 0.0409. To compare, dry matter of wild-type plants grown under control conditions for roots: 0.0466 ± 0.0093; for shoots 0.2003 ± 0.0340.

Achievements in relation to the state-of-the-art

The novelty of the research performed consists in using for transformation specific genes, newly cloned - and in applying comparative approaches:

(1) Expressing two homologous genes with the same physiological function (control Zn root-to-shoot translocation) in the same species to conclude on similar/different effects;

(2) Expressing the same gene in two plant species (tobacco and tomato) - to investigate whether the same construct may be widely used to get the same qualitative and quantitative modification of Zn and Cd content (to improve Zn and reduce Cd content);

(3) Using a strong native promoter for genes cloned from Zn-hyperacumulator *A. halleri* in addition to a strong constitutive CaMV promoter.

Brief summary of the major achievements:

(i) The most significant enhancement of Zn translocation to the shoots (up to 3-fold higher Zn concentration in shoots of plants) was noted in both tobacco and tomato expressing *HMA4* (both *AtHMA4* and *AhHMA4*). Moreover, there was Cd reduction in both roots and shoots of tobacco expressing either *AtHMA4* (Fig. 1, 2) or *AhHMA4.* However, in tomato expressing either of these two constructs, no decrease of Cd level in transgenics was found.

(ii) Substantial increase in Zn level in the shoots was detected also in tobacco expressing *AtHMA4*-C-terminus grown at low 0.5 µM Zn (**Figure 2**) (C-terminus contains numerous cys and his metal binding residues, and as our GFP study showed it is localized in the cytoplasm).

(iii) Other genes used for the transformation did not contribute to the modifications of Zn and Cd accumulation to the extent which would meet the biofortification needs.

(iv) It is worth to note, however, that expression of *AtMRP7* resulted in the enhanced Cd tolerance, Cd accumulation in vacuoles and Cd retention in the roots, which has the potential biotechnological application - it is promising for the phytostabilisation. Moreover, expression of *AtECA3* contributed to enhanced tolerance to Zn and Mn excess, accompanied by the reduction of the accumulation of these elements.

(v) In all transformants, the pattern of metal accumulation depended strongly on metal supply.

Conclusions

(i) The use of *AtHMA4*, *AtHMA4*-C-terminus and *AhHMA4* is recommended for Zn-biofortification purposes.

(ii) The phenotype of transformants depended on the external metal concentration; it thus might be difficult to engineer a plant displaying the desired metal-related phenotype, when grown under conditions of metal-supply varying from severe deficiency to metal excess. Thus the expression of a transgene does not fully overcome the internal metal-homeostatic mechanisms. The dependence of metal accumulation on a plant metal-status is likely related to a plant-endogenous homeostasis mechanisms, specific for low and high metal-status, which to a different extent counteract the effects of a transgene expression.

(ii) Expression of the same gene / construct in different species does not necessarily lead to the same pattern of the modification of metal partitioning. The knowledge on the interaction between a transgene and an endogenous homeostatic system of a host plant is so far very limited, however, likely decisive in the generation of the phenotype of a transgenic plant.

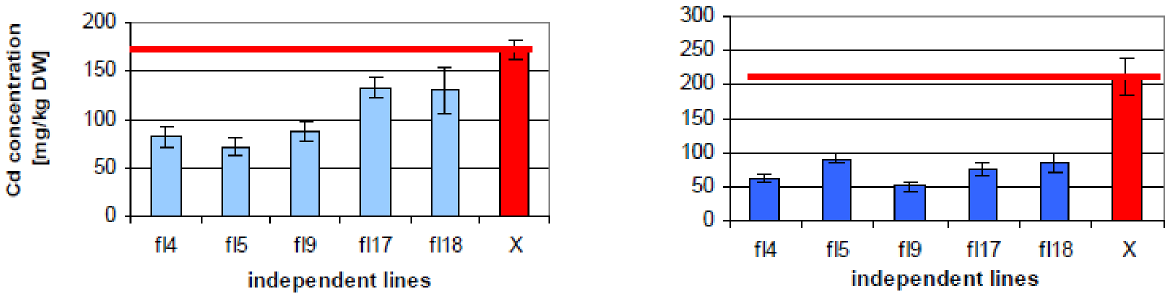

Figure 10. Cd concentration in the shoots and the roots of 5-week old AtHMA4-expressing tobacco and the wild-type grown in ¼ Knop's medium and exposed for 4 days to 0.25 µM Cd. X=WT (wild-type); numbers on the x axis = numbers of independent transgenic lines.

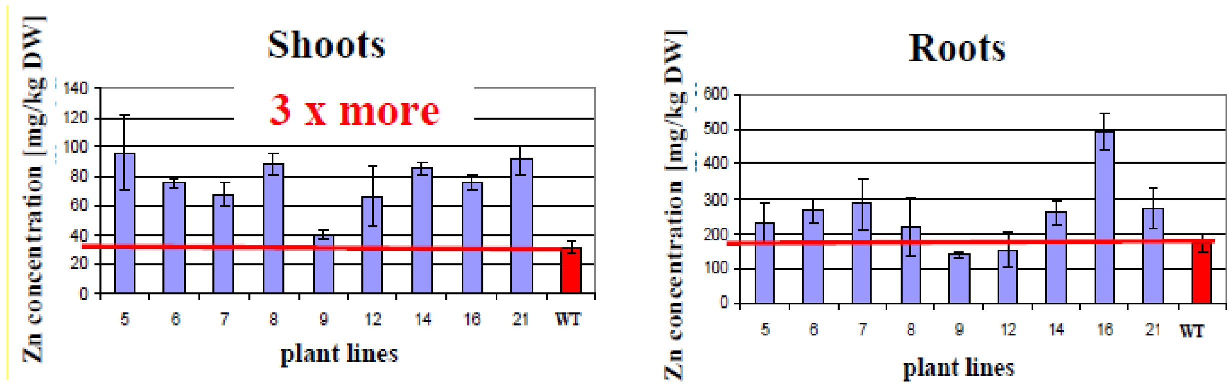

Figure 11. Zinc concentration in the shoots and the roots of AtHMA4-C-terminus expressing tobacco and the wild-type grown for 2 weeks in ¼ Knop's medium containing 0.5 µM Zn. WT = wild-type) ; numbers on the x axis = numbers of independent transgenic lines).

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Workpackage IV:3:4 Testing of plant varieties under glasshouse and field conditions

Summary description of the WP objectives

Growth conditions dictate the physiology of all plants. It has therefore been essential to test how natural conditions influence the response of barley, tobacco and tomato to heavy metals. The transgenic plants generated in this Project, however, were tested under field conditions. They have been grown in classified green houses, strictly according to current safety regulations.

The goal of this WP was to establish methods for diminishing heavy-metals uptake and distribution in plants grown in the field. The achievements lead to better understanding of good agriculture practices.

Metal ions have to travel long way from the soil before they enter edible parts of the plant. The process of biofortification of metals is related to the soil properties, level of bioavailable fractions of metals as well as to the genetic features of plant species, cultivars and lines.

Aims of the barley studies included:

- Screening for barley cultivars accumulating Cd below the limit value of 0,1 mg kg^{-1}w.w. (0.116 mg kg^{-1} d.w.);
- Assessment of Cd concentration in barley grains in context of soil type (texture) and metal content;
- Search for soil factors influencing Cd accumulation in barley grains;
- Soil metal stabilization using zeolite (1 and 3%) and organic matter (lignite) to abate Cd grain concentration;
- Spraying leaves with Zn for diminishing Cd grain concentration; Influence of fungicide application on Cd grain concentration;
- Influence of different fertilizer application (on leaves and/or soil) on Cd grain concentration;
- Influence of mycorrhiza on Cd concentration in grains;
- Evaluation of the utility of dwarf and short-root barley mutants as a source of hypo-Cd-accumulating plants.

Contractors involved

IETU (WP leader), UBT, KVL.

Methodolgies and approaches employed

The plants generated in SP IV:2 and SP IV:3 were investigated in detail under various conditions. Field experiments with natural ecotypes involved selected polluted sites in Poland, whereas experiments with transgenic plants took place in pot assays in classified greenhouses. In order to investigate the natural variation among cultivars, non-transgenic crop cultivars were tested at industrial waste sites, primarily contaminated by Cd and Ni. Soils from these sites were transported to contained green-house experiments involving transgenic plants. Plant parts and soil samples were collected for analyses of heavy metals by ICP. Field biomasses were correlated with the exclusion of metals.

Further, the relation between root arbuscular mycorrhiza (AM) and Cd grain concentration has been evaluated in barley cultivars, as well as an evaluation of fungicide application on frequency of root AM and Cd grain concentrations in this crop plant.

Interaction between Partners and WPs

There has been a close co-operation between partners and with other WPs within the Pillar IV. Partners, mainly South, LIP, MPI and UZH (SPs IV:2 and IV:3) were involved in the identification of genes of particular importance for heavy-metal transport and accumulation in plants. They were initially characterised by complementation in microorganisms. Promising candidate gene constructs prepared by Partners were sent to WarsU for plant transformation and initial characterisation of received independent lines with respect of their ability to take up metals (WP IV:3:3). Seeds of T_0 generation were sent to IETU (WP IV:3:4) for pot experiments to investigate metal accumulation in parts of plants cultivated on contaminated soil.

Achievements in relation to the state-of-the-art

Up to date, correction of pH was treated as routine activity for the site, not as hot spot activity. Application of fungicides may be responsible for higher Cd accumulation in grains/cereals.

Cd grain concentration vs. soil properties and contamination - Investigations have shown the influence of pH on Cd grain concentrations in barley cultivars. On the field, soil properties vary from place to place. Uneven distribution of pH values on site is often referred as a mean value, whereas metal uptake is related to the point scheme. It is why higher concentrations of heavy metals in plant samples may be found on clean soil.

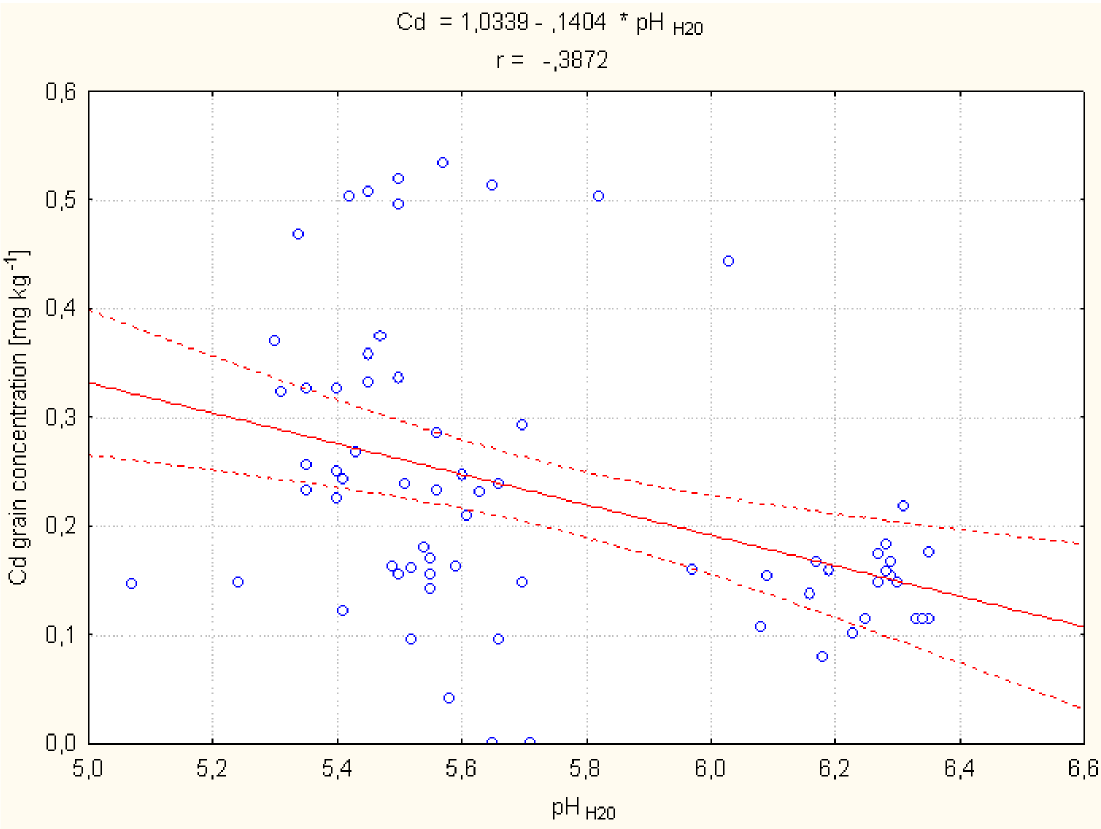


Figure 12**. Relationship between soil pH and cadmium grain concentration.**

Significant correlation between Cd concentrations in grains and the EC (ions competition) was found as well as significant negative correlation between Cd grain concentration and pH. Three of Polish barley cultivars, the *Carola*, the *Gregor* and the *Traminer* may be considered for contaminated areas, when earlier immobilization of bioavailable forms of Cd is provided. Even though in those cultivars limit values for consumption are exceeded, they may be used for feed purposes.

Soil metal stabilization by organic matter addition vs. Cd grain concentration- Soil contaminated with heavy metals may be remediated with application of organic matter e.g. lignite (brown coal) or molecular sieves as zeolites. Decrease of metal concentrations in barley grains after addition of lignite and zeolite to the contaminated soil could be observed e.g. a 3% decrease in the Nagradowicki cultivar.

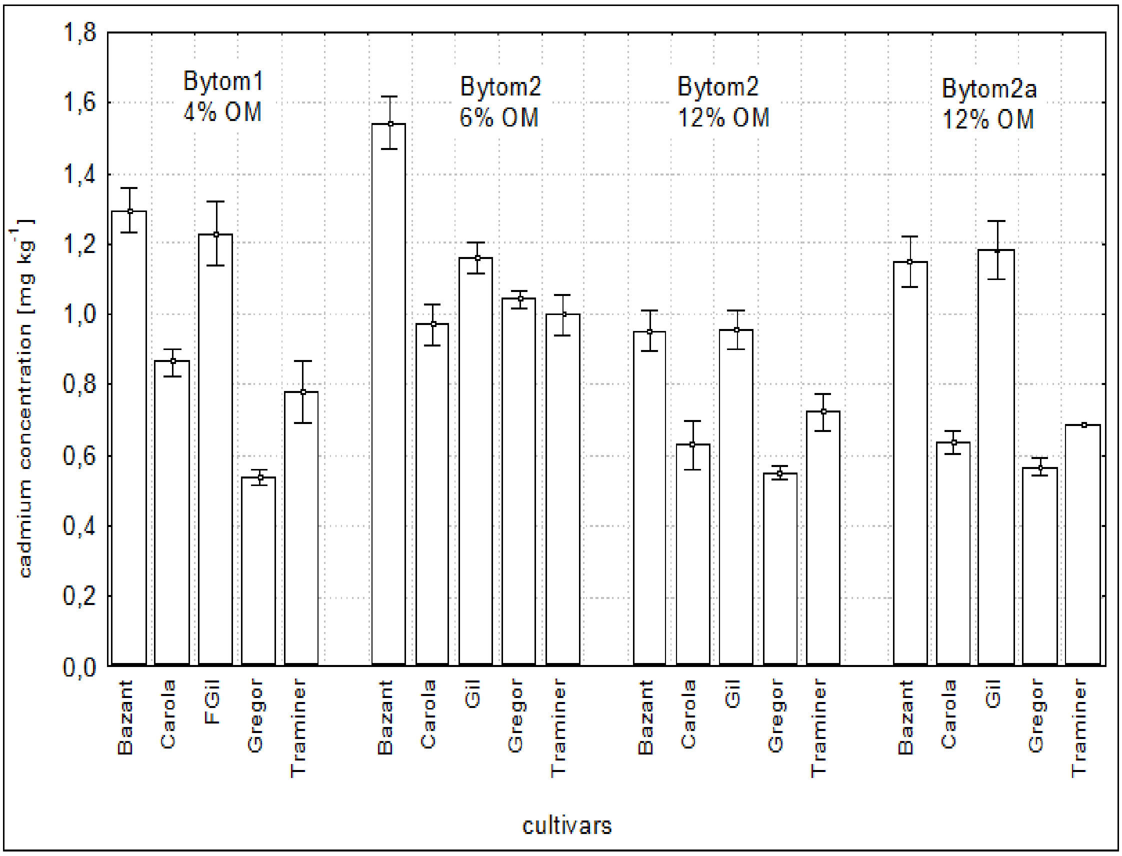


Figure 13.Cadmium concentration in barley grains depended on additives, cultivars and sites.

Cd concentrations in barley grains after overhead Zn spraying - Overhead Zn application during milk phase of barley growth results in reduction of Cd concentrations in grains. Zn spraying also significantly reduces Cd content in barley straws.

Cd grain concentration vs. fungicide foliar application or NP soil fertilizer - The use of fungicide for plant protection and NP soil fertilizer affect the occurrence of mycorrhiza. Significant negative correlation between mycorrhization frequency and Cd grain concentrations was revealed. Among other, for Polish barley cultivars *Carola* and *Bażant* mycorrhizal colonization (F%) decreased with increasing concentrations of Cd in soil.

Plant protection with fungicide (AMISTAR) against fungi may result in statistically significant increase in Cd concentrations in barley grains. Two weeks after AMISTAR application lack of mycorrhiza in roots of studied plants was observed. The same effect was noticed after application of NP-fertilizer to the soil what may be related to the impact of phosforus excess on diminishing frequency of mycorrhization.

Utility of dwarf and short-root barley cultivars (originated from mutagenesis) as a source of hypo-Cd-accumulating plants.- Dwarf mutants that accumulated higher amounts of Cd than limit values were investigated. Low numbers of root hairs were positively correlated with Cd concentrations.

Genetic modifications vs. Cd fruits concentration - The significant decrease in Cd concentrations in tomato fruits was observed only for genetically modified line MPR-15, comparing to mother line (MM).

Differences in Cd concentrations in tomato fruits from other genetically modified lines were not significant. Positive, medium level of correlations, between concentrations of Cd *vs.* Fe , Zn *vs.* Fe (r = ,63) and Cd *vs.* Zn (r = ,67) were found.

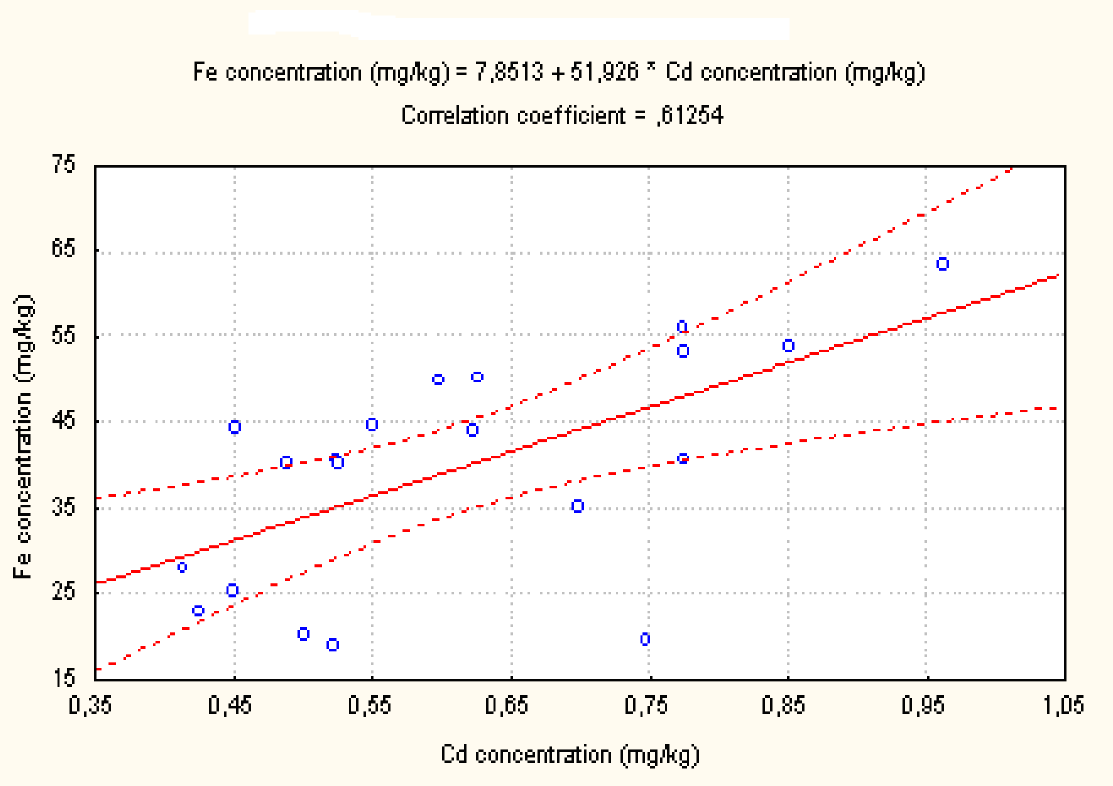


Figure 14. Correlation between cadmium and iron in transgenic tomato fruits.

Genetic modifications vs. Cd leaves concentration - Genetically modified tobacco grown on clean soil can accumulate lower amouts of Cd compared to the mother line. The significant decrease in Cd concentrations in tobacco leaves was observed for three transgenic lines comparing to mother line (Xanti).

Conclusions

Species and cultivars of a plant play a crucial role in heavy metal uptake and distribution within different organs.

So-called clean soils, with heavy-metal concentrations below the limits, may activate processes that lead to accumulation of metals in edible parts of barley. Any mechanism for increasing the pH values in soils lead to stabilization of metals, preventing their uptake by plant.

One approach to this problem is to develop cultivars that accumulate lover levels of Cd in grains. Therefore, the present strategy should be to avoid the excess exposure to Cd by choosing clean soils for agricultural purposes, concomitantly with constant monitoring for soil properties.

Global deliverables of Pillar IV

Zinc biofortification of cereals: problems and solutions

Overall conclusions

The goal of biofortification is to develop plants that have an increased content of bioavailable nutrients in their edible parts. Cereals serve as the main staple food for a large proportion of the world population, but have the shortcoming, from a nutrition perspective, of being low in zinc (Zn) and other essential nutrients. Major bottlenecks in plant biofortification appear to be the root-shoot barrier and - in cereals - the process of grain filling. Our new findings demonstrate that the root-shoot distribution of Zn is controlled mainly by heavy metal transporting P1B-ATPases and the metal tolerance protein (MTP) family. A greater understanding of Zn transport will be important to improve crop quality and also to help alleviate accumulation of any toxic metals. The major molecular results are summarized in **Figures 15** and **16**.

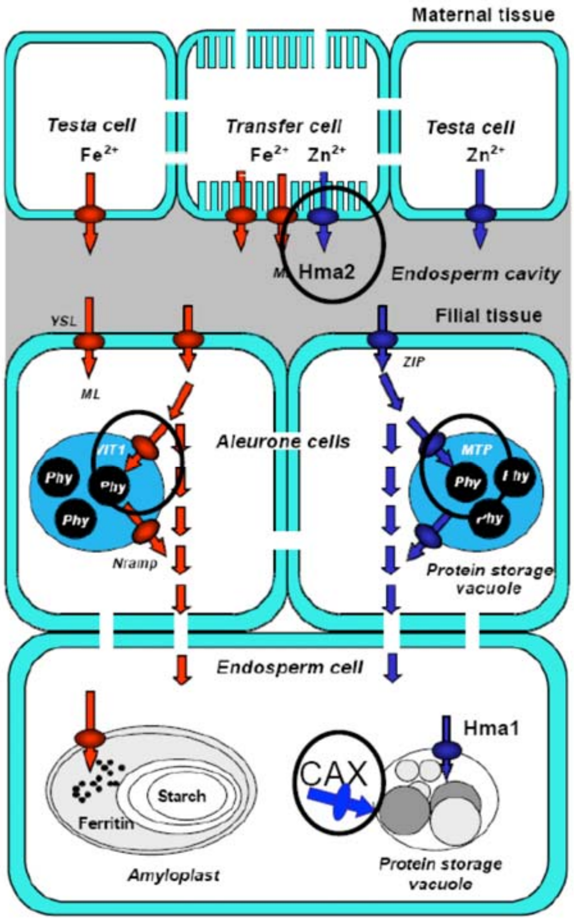
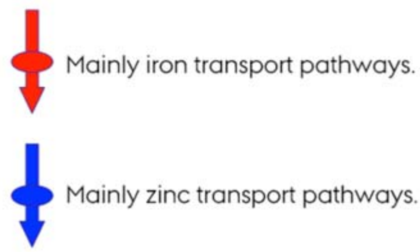


Figure 15. Transport proteins identified with a likely role in heavy metal loading into the cereal grain. Phy: phytate; ML: Metal ligands like metallothionein, citrate or nicotianamine; VIT: Vacuolar iron transporter; Nramp: Iron ellux transporter; MTP: Metal Tolerance Protein; ZIP: Zinc Influx Transporeter; YSL: Yellow Stripe1-like Transporter.



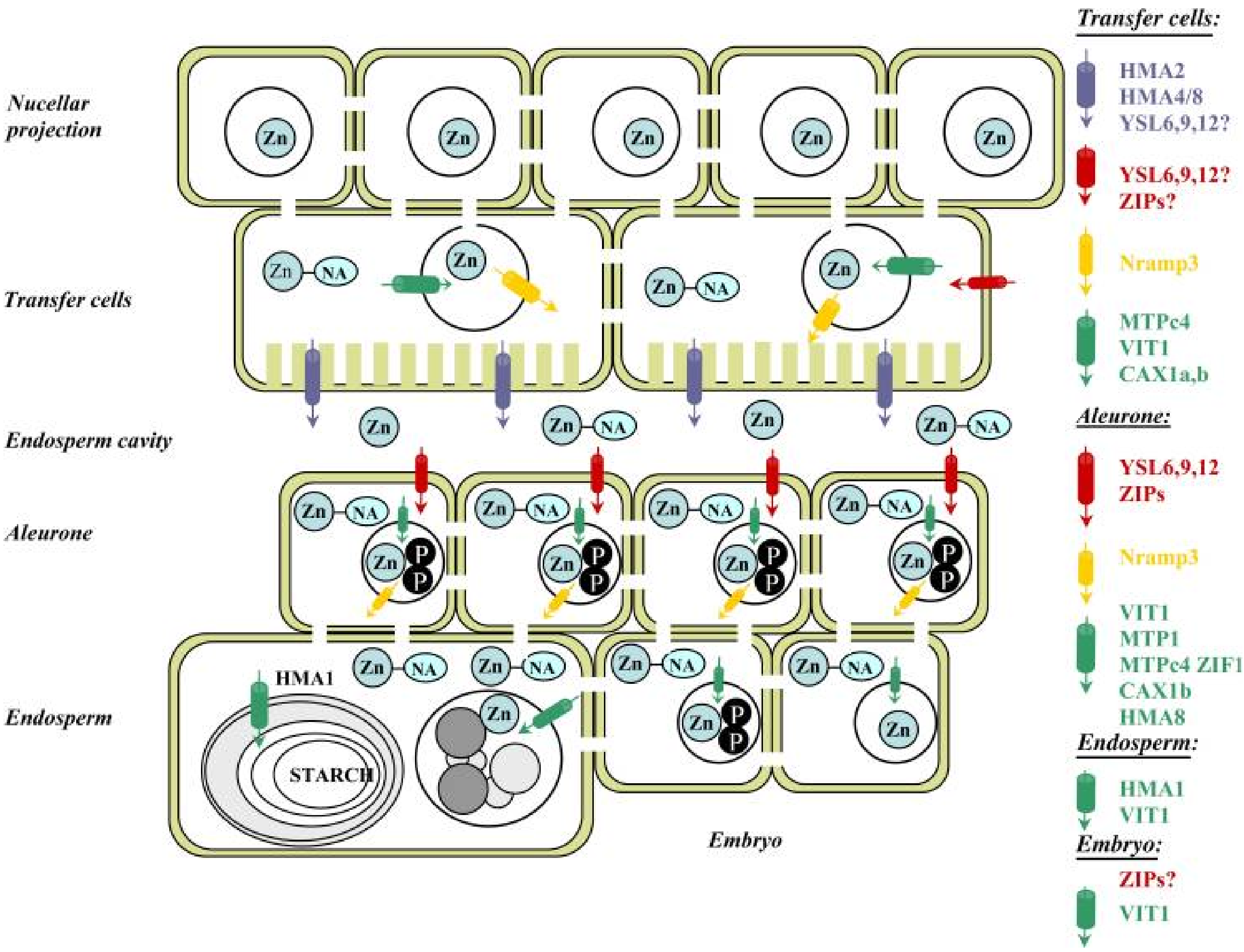


Figure 16. Proposed road map for Zn trafficking from the phloem to the final sequestring sites in the developing grain. Blue cylinders indicate cellular efflux transporters, red cylinders indicate cellular importing transporters, green cylinders indicate vacuolar uptake transporters, and yellow cylinders indicate vacuolar efflux transporters: Zn, zinc; NA, nicotianamine; P, phytate.

What are the solutions?

A breeding approach to produce nutritionally improved cereals relies on genetic diversity in natural populations that can be crossbred to introduce traits/genes from one variety or line into a new genetic background. There is limited documentation for the natural diversity in grain Zn content of cultivated wheat or rice varieties. More primitive varieties, however, can be a valuable genetic resource. Gpc-B1 (GRAIN PROTEIN CONTENT B1) is a wheat quantitative trait locus associated with increased grain protein, Zn and iron (Fe) content. Introduction of the Gpc-B1 locus from *Triticum turgidum ssp. Dicoccoides* into different recombinant chromosome substitution lines results in higher concentrations of Zn, Fe, manganese (Mn) and protein in the grain, compared to the concentrations in lines carrying the allele from cultivated wheat.

Increased metal content is associated with earlier senescence, but it remains to be investigated whether the introduction of the Gpc-B1 locus influences grain yield negatively. Lower yields are not likely to be an attractive solution to the micronutrient problem for farmers and for the world’s population. Moreover, lower yields would imply that total amounts of micronutrients loaded into the grainsof Gpc-B1 wheat are not enhanced. Information on other loci influencing micronutrient grain content is scarce. To learn more about the genetic basis for cereal grain metal contents, an obvious approach will be to analyze genetic diversity in model cereals, such as rice and barley.

A transgenic approach to increase the Zn content of cereal grains might involve the manipulation of transporters involved in Zn translocation. With respect to Zn uptake, translocation and deposition, a predominant role seems to be played by members of the ZIP family and, at least in dicots, the MTP family. Furthermore, with respect to root-to-shoot allocation of Zn, the Zn pump HMA4 seems to be a major player in dicots. It remainsto be tested whether HMA4 might be used for transgenic biofortification approaches in cereals. To eliminate the need for transgenes, it might be possible to achieve the same results by targeted molecular breeding. A more conventional approach would be to increase the use of Zn fertilizers (fortification) to overcome the problems of Zn deficiency. However, although application of Zn fertilizers should potentially reduce grain Cd concentrations, this depends on the concentrations of Cd in fertilizers, which indeed remain a major source of cadmium (Cd) taken up by crops.

Further clarification of Zn transport and chelation mechanisms, especially of those that might be specific for monocots and cannot be inferred from work on *Arabidopsis*, is a major task at hand. The clarification of these mechanisms might eventually allow a focused strategy for improving the Zn content of cereal grain via the enhancement of Zn uptake, transport and remobilization. Manipulation of Zn transporters and Zn ligands in the aleurone and the embryos might increase the Zn content of the globoids. In this context, future research should also address the possibility of modifying ion selectivity of Zn transporters, for example, to enhance Cd sequestration in root vacuoles. The general feasibility of such approaches is evident from a study in which a single amino acid substitution of the IRT1 transporter from *A. thaliana* abolished its affinity for Zn, while preserving the Fe uptake ability. An alternative to reducing Cd in the plant would be to express Cd exporters at the root level.

Section 1.5

Horizontal WPs

WP C:1 Quality control, quality assurance and validation

Summary of WP objectives

i) To organize quality control (QA)/quality assessment (QC) systems for acquisition of comparable and traceable data with stated uncertainties; obtained under quality conditions in all WPs dealing with element determinations and analyses of other biomarkers of exposures and effects;
ii) To document and give access to the questionnaires for assessment of background data, exposure determinants and symptoms and signs of disorders (in particular neurobehavioural testing).

Contractors involved
IJS (WP leader Milena Horvat), All Partners.

Degree to which the objectives were reached
The objectives have been achieved.

Methodolgies and approaches employed
Achievements in relation to the state-of-the-art
Hence, WP C:9 was involved in most of the WPs in Pillars I, II, and III, and some in Pillar IV. The WP has supervised all aspects of QC/QA within PHIME, in agreement with internationally accepted protocols. Thus, QA/QC represented an integrate part of all PHIME WPs that included chemical and biological measurements, as well as questionnaires and examinations. Validation of all the methods and the harmonisation of protocols have been performed. This was absolutely necessary to have full integration and to make possible merging of materials and conclusions, based upon different studies within PHIME.

Standard operation procedures
Issues on standard operation procedures (SOPs) for sampling and sample handling were discussed and compiled. Experience was continuously shared between Partners.

Inter-comparisons of element determinations
This included regular participation in inter-laboratory studies and proficiency-testing schemes, organised at national and/or international level. Recognized schemes at national and international level were used. Further, the use of PHIME reference materials (fresh and freeze dried) for blood was encouraged and the QC charts recorded. Inter-comparisons were made, reported and discussed at the Annual Meetings of PHIME. Usefulness of commercially available reference material (Seronorm), and the participation and performance in international external QC schemes were critically evaluated. Based on the outcome of the PHIME inter-comparisons, statistical power analyses were done, to estimate the minimum number of samples needed for human biomonitoring planned in Pillar III. Strategic issues, such as centralizing of certain analyses to one laboratory were considered and solved.

Genotyping
QC/QA activities in genotyping are discussed below (WP C:2).

Nutritional biomarkers
A large number of nutritional and effect markers were analyzed in the framework of PHIME. The methods were compiled and distributed among the Partners.

Questionnaires
Several WPs developed questionnaires as a basis to acquire basic background information, exposure determinants, as well as symptoms and signs of disorders. A compilation of tools was made available to all WPs.

Testing of function/disorders
Several WPs within PHIME used tests of organ functions. Hence, methods for neurobehavioural testing was discussed and coordinated within Pillar I. Further, neurophysiological testing of peripheral nervous system was an issue between Partners in WP III:1. Also, testing of kidney function was an issue in several of the Partners in Sub-Pillar II:3 and WP III:2.

Conclusions
Though, of course, known already before start of the Project, the PHIME activities fully illustrated the absolute need to carefully supervise QC/QA activities. Thus, large efforts were spent on such issues. One of the most striking observations was that even experienced laboratories had problems in element determinations, when there was a need of sufficiently good accuracy to allow merging of information from different labs, and when there was a need to acertain limited differences between study groups. This awareness has been carefully dissiminated within PHIME, and transferred from PHIME to new EU projects, which have to address similar problems (e.g., ESBIO, DEMOCOPHES, COPHES).

Thanks to the efforts in WP C:9, it has been possible to merge information from different Partners to an extent only occasionally possible in wide international collaboration.

WP C:2 Gene-environment interaction

Summary description of WP objectives
The aims of the WP comprise:
i) To support the PHIME partners in design of gene-metal interaction studies, performance of molecular genetic analysis, and statistical analysis of gene-metal interactions**;**
ii) To do the assay designs as well as laboratory analysis of genetic markers of relevance for the metals analysed**;**
iii) To be responsible for the QC/QA of molecular genetic analyses performed;
iv) To establish a polymorphism database for the markers and populations analysed within PHIME;
v) To organise training and workshops in gene-metal interactions for the PHIME Partners.

Contractors involved
LU (WP leader Karin Broberg), UKU, Sosno, FUDAN, ICDDR,B, KI, ChildH, Bresc, MOHS, UUC, and UNIUD.

Degree to which the objectives were reached
The objectives have almost entirely been achieved.

Methodologies and approaches employed
The support of the Partners for studies of gene-environment interactions has mostly been through on-site meetings, video conferences, and through e-mail. The polymorphism selection and the assay design have been aided by literature and database searches (Pubmed, Hapmap, NCBI dbSNP, Ensembl, etc.), as well as by the results presented by Pillar IV Partners in PHIME. For the genetic analyses, different methods have been used within PHIME: From low throughput using the multiplex PCR or restriction fragment length polymorphism assays, medium throughput using the Taqman assay or the Sequenom, to high throughput using the genome wide microarrays. For the QC/QA work, guidelines for performance of the genetic analyses have been set-up for the Partners. Inter-laboratory comparisons, within PHIME, as well as outside, have been made for assays using restriction fragment length polymorphism analysis and Taqman technology.

Training has been performed by a one-week theoretical and practical course in Lund (Partner LU), addressing gene-environment interactions, lectures during courses in metal toxicology/environmental health in Medical School, University of Brescia (Partner Bresc), several workshops during the Annual Meetings, as well as on-site two weeks-to-month visits by PHIME younger scientists (N=9) at the molecular genetics laboratory of LU. Partner LU has also visited all labs that have performed genetic analyses within PHIME.

Achievements in relation to the state-of-the-art
There is a large difference in the risk of toxic effects of mercury, arsenic, cadmium and lead between individuals. The genetic background is probably of importance for the differences in susceptibility, but not much has been known earlier. In PHIME, we have so far been able to establish the role of the *AS3MT* genotype as a key determinant for arsenic metabolism, *ALAD* genotype for neurotoxicity of lead, and *MT1A* genotype for kidney toxicity of cadmium, and more results are to come. This has been possible through close collaboration between the different PHIME partners and exchange of knowhow in metal metabolism/toxicology, molecular genetic analysis and biostatistics, and samples for analysis.

Conclusions
Extensive actions hve been taken within PHIME to disseminate methods on genotyping and interpretation of results. As to novel scientific information, we have been able to demonstrate gene-environment interactions for arsenic, mercury, cadmium, lead and manganese (data and publications presented under respective WP, above).

WP C:3 Epidemiology, biostatistics, and risk assessment and public health aspects

Summary description of WP objectives
The main objectives were:
(i) To ascertain that appropriate study designs and adequate statistical analyses are applied for the epidemiologic studies within PHIME;
(ii) To promote discussions on how to strengthen the reporting of epidemiologic studies in scientific journals;

(iii) To propose adequate methods for risk/benefit assessments and public health implications.

Contractors involved
LU (WP leader Ulf Strömberg), All other Partners in Pillars I-III.

Degree to which the objectives were reached
The objectives were achieved.

Methodologies and approaches employed
Achievements in relation to state-of-the-art
The WP leader has organized five workshops on epidemiological study design issues and statistical methods in connection with the kick-off meeting and the following four Annual Meetings. Major focus was on multivariate methods for analyzing data from epidemiologic studies. The response from the workshop participants has been very favorable. On the other hand, electronic communication with the members of the WP C:3 Working Party (17 members, representing different Partners), which was established early in the Project period, has led to little response and circulation. Therefore, during the second half of the project period, the WP leader decided not to pursue communication of statistical issues (of general interest for the PHIME researchers) restricted to the Working Party members only.

The workshop *Strengthening Scientific Reporting,* held at the Third Annual Meeting in Lund (about 80 researchers participated), was well appreciated. This workshop prompted insightful discussions based on the published recommendations for reporting of epidemiologic studies in journals (www.strobe-statement.org) and the coming additional recommendations for molecular epidemiology studies (STROBE-ME).

The "wrap-up" work on the fish-intake related biomarkers hair-Hg and S-PUFA (i.e., long chain n-3 polyunsaturated fatty acids measured in serum) and their impact on the myocardial infarction (MI) risk, using pooled data from Sweden and Finland, provided a valuable exercise; we thereby gained knowledge on adequate methods for risk/benefit assessments (Wennberg et al., submitted). The results were obtained by careful multivariate statistical modeling. Fractional polynomial modeling turned out to be useful for approximating the association between the fish-intake markers hair-Hg and S-PUFA (combined) and MI risk. Moreover, based on the preferred model, we were able to estimate the expected preventable fraction of cases, which reflected the potential public-health impact of achieving a systematic change in the biomarker distribution for a population.

Public-health issues have been central in many WPs in Pillars I-III. Hence, such issues have been frequently discussed in many WP and Pillar meetings. In this connection, a working party containing Members from all three Pillars are compiling PHIME and other information on long-term, low level cadmium exposure, in order to arrive at a paradigm change in risk assessment from subtle renal tubuli effects to other impairment, in particular the bone effects focused in Sub-Pillar II:2 (Åkesson et al., manuscript).

Conclusions
It was, indeed, very important to ascertain that appropriate study designs and adequate statistical analyses were applied within the PHIME Project, where several epidemiologic studies have been carried out. Statistical method issues, and scientific reporting recommendations, were communicated to the members of PHIME by the workshops held,

which were successful and well appreciated by the Partners. The other way of interaction between the PHIME Partners, i.e., with an early established WP C:3 Working Party, led to little response from the members; the intention of a creating an active Working Party was not fulfilled.

The risk/benefit assessment work, based on the pooled data on two fish-intake-related biomarkers (hair-Hg and S-PUFA) and MI outcomes from Finland and Sweden, was fruitful. Methods for such data analysis were suggested and applied successfully. Also, an article, using PHIME (and some other) data as a basis for a change of paradigm in risk sassement of cadmium has been prepared.

Publications

Åkesson A, Bergdahl IA, Barregård L, Nordberg M, Skerfving S, Nordberg G. Environmental cadmium exposure in the general population – time for a shift of paradigm in its risk assessment? Manuscript.

Wennberg M, Strömberg U, Bergdahl I A, Jansson J-H, Kauhanen J, Norberg M, Salonen J T, Skerfving S, Tuomainen T-P, Vessby B, Virtanen J K. Myocardial infarction in relation to mercury and fatty acids from fish: A risk-benefit analysis based on pooled Finnish and Swedish data in males. Submitted.

WP C:4 Nutrition and toxicology: an integrated approach

Summary description of WP objectives

The subjects of nutrition and toxicology have developed separately into two independent research and teaching disciplines, focussing on the beneficial and the negative effects, respectively. However, many examples attest to the importance of interactions between dietary components and toxicants after absorption in the body. The challenge of regulators can be to weigh the balance between risks of deficiency of a metal with that of toxicity. Or the benefit of a nutritious food-stuff with that of its contamination with a toxic agent.

The aim of this WP at the outset was to bring together nutritionists and toxicologists who have an overlap of interests with the aim of stimulating discussions and integration between disciplines. However, as reviewers proposed that Pillar IV would be integrated into this work package the scope was widened.

Contractors involved

UnivRoch (WP Thomas Clarkson), UUC, LU, All other Partners (Pillar IV was, from the outset, not planned to be involved).

Degree to which the objectives were reached

The WP has been very successful in stimulating interest and cooperation across disciplines. This is true not only for those intended at the outset of the Project (nutritionists and toxicologists), but also for plant physiologists of Pillar IV.

Methodologies and approaches employed

Interdisciplinary workshops/seminars.

Achievements in relation to the state-of-the-art

A first seminar clearly showed the importance of considering interactions between nutrients and toxic elements. Hence, interaction between mercury, long-chain n-3 polyunsaturated fatty acids and selenium are crucial for the interpretation of effects on the central nervous and the cardiovascular systems. Also, for the toxicity of arsenic, protein, folate and vitamin B12 intakes may interact. Thus, the PHIME WPs should consider the status of their study populations as to the status of these nutrient, as well as iron, calcium and iodine. In addition, the Partners became more aware that, interactions between the toxic elements (mercury, cadmium, manganese, lead and arsenic), as well as other toxic agents (e.g. persistent organochlorine pollutants), should be more closely addressed.

At a second seminar biofortification and soil and plant bioavailability issues were discussed in depth.

At a following workshop, talks were given by nutritionists and toxicologists also plant bioavailability issues were discussed in depth. The focus this time was on uptake of metals in the food chain. As the latter aspect turned out to be important it was also addressed in the subsequent workshop. Prof. Ismail Cakmak from Sabanci University gave an overview of newest research on the counteraction of zinc and cadmium uptake depending on fertilization. Dr. Stefan Fabiansson from the European Food Safety Authority (EFSA) explained the official opinion on cadmium exposure for the European Population. Dr. Agneta Åkesson from Partner KI presented research results on cadmium and cancer. The final speaker, Dr. Jonas Tallkvist from Swedish Agricultural University, discussed the intestinal uptake of heavy metals.

The presentations at the final workshop covered risk assessment, use of biomarkers, identification of groups with increased exposure, and carcinogenicity. In addition the partners discussed wrap-up activities (integrated activities of several work packages). All presentations were followed by lively discussions.

Conclusions

Creating this interest in this WP seeing the broader picture has required enthusiasm and insistent work. There was a possibility to integrate proposals from the reviewers. Therefore, the work within this WP took a somewhat different course than planned at the beginning. The role of WP leader was, during the course of the Project, more and more taken over by Partner LU.

This WP was a valuable resource to exploit/interact with/get inspiration from and between disciplines within this heterogeneous project. Also the Scientific Advisory Board added valuable input. Furthermore, this WP gave the opportunity of involving researchers from other EU projects.

WP C:5 Training

Summary description of WP objectives
To achieve exchange of young scientists, transfer of knowledge and methods among Partners by organizing training courses for PhD students and postdocs.

Contractors involved
Bresc (WP leader Roberto Lucchini).

Degree to which the objectives were reached
The objectives were fully reached.

Methodologies and approaches employed
The training program has been conducted during 2008. The first part included the organization and preparation of the scientific content of a training course. This was constructed with the aim of training PhD studens and and postdoc, according to the different topics covered by the PHIME project. Therefore, most of the WP leaders were invited to participate in the course. Risk assessment of metal exposure was chosen as the course general title, to offer the participants the background knowledge for preventive strategies. The selected topics included updates on the toxicology of mercury, arsenic, cadmium, lead, manganese, exposure sources and levels and related health effects. The course covered also the main methodological aspects of epidemiology, exposure assessment, gene-environment interaction, statistical model for complex datasets and risk assessment procedures.

The second part of the WP was dedicated to the participants' enrolment. Invitations were sent to all PHIME participants and the course announcement was posted on the public section of the PHIME website www.phime.org. Applications were sent from many parts of the world and a total number of 48 were accepted. The course took place at the Medical School of the University of Brescia, Italy, from 18 to 26 September 2008. The activities were diversified between lectures in the mornings and group work in the afternoons, when students were given titles by the speakers to be further developed, presented and discussed by the students. All teaching materials were collected in electronic format and distributed to the participants.

Additionally, on 29 September to 2 October, 2011, a Summer School course (Occupational and environmental determinants of disease:multidisciplinary approach as a key for research and prevention) was organized by Partner Bresc, and held at Medical School of the University of Brescia, Italy. Three PHIME WP participants lectured on metals exposure, manganese toxicity and gene-envrironment interaction.

Conclusions
The training course was quite successful in terms of the quality of the lectures, and students' active participation. Evaluation forms were filled out by the students, who generally appreciated the good quality and usefulness.

The activities were conducted within the timeframes that had been indicated, and were advertised properly enough to reach the target number of participants. Logistic were prepared timely and travel funds and lodging was provided to all participants. All of these were important factors, especially for students from third countries.

WP C:6 Gender aspects

Summary description of WP objectives
Main objectives of the Gender activities were to make both the PHIME management structure and the research activities proactive as to gender aspects, and to raise awareness among all Partners on gender aspects among researchers, in management and in the research.

Contractors involved
All Partners were involved. WP leader was UmU (Ingvar A. Bergdahl).

Degree to which the objectives were reached
The original male dominance in the management of the project was decreased and in the health-related studies gender differences have been, throughout the planning and execution of the Project, an integrated part.

Methodologies and approaches employed
Assistant Leaders Programme: In order to train and support researchers for leading positions, each Member of the initial Project Coordination Committee had an Assistant Leader, in most cases of the opposite gender. This resulted in recruitment of five female Assistant Leaders, who were regarded as advanced trainees and participated in management meetings.

Gender issues integrated in the research: Gender was an integrated part of the research from the planning and throughout its execution.

Awareness raising and FP6 networking: Workshops specifically targeting gender issues were held at the First and Fourth Annual Meetings. PHIME participated in FP6 Gender meetings and activities.

Achievements in relation to the state-of-the-art
Assistant Leaders Programme: The appointed Assistant Leaders participated in the management meetings (Project Executive Committee) and formed a group for internal networking. Early in the Project they expressed that they wanted to:

- Interview Pillar leaders and other senior researchers about career advancement/leadership strategies;
- Become more visible at meetings, get a more important role;
- Attempt to benefit from the mentor-like role of the Assistant Leader.

Plans of involving in leadership training courses or workshops, on, e.g. 'Academic leadership' or 'Women in leading positions', were abandoned after discussions with senior researchers. The Assistant Leaders decided to rather focus on successful publishing, proposal writing, obtaining good CVs, and be more visible. Two of them advanced in the Project to position as Pillar leaders, when it was decided to split Pillar II into three Sub-Pillars. Eventually, four out of the five Assistant Leaders remained in the Project until the end of it, all of them with good progress.

Gender issues in the research: Most of the scientific work on gender issues was integrated in the different WPs. In addition, a workshop on "Gender differences in exposure and effects of metals" was arranged during the Fourth Annual Meeting. An aim was to highlight the potential in studies of gender differences in the data materials within the Project, so that opportunities to do gender studies were not overlooked. Seven researchers gave brief descriptions of the most important findings on sex/gender differences and similarities within their studies, and we could note that also those who had not worked much on gender differences could present well-founded hypotheses, though sometimes the statistical power or design was not optimal for studies of gender issues. One problem in epidemiology is that studies of gender differences often require doubled size of the study, and the size of the study is often the cost-limiting factor. Therefore, advancement in this area is dependent of funders realizing its importance.

Networking in FP6: Unfortunately, the previously existing arrangements by the Commision for networking between FP6 projects on gender issues were interrupted, and attempts to collaborate on gender issues with other projects were unsuccessful. The Assistant Leader Programme could possibly have benefited from such networking.

Conclusions
The model with Assistant Leaders of the opposite gender resulted in a programme that appeared useful. Much of the PHIME research has considered gender differences. Since the size of an epidemiologic study often determines its cost, and studies of gender differences often require doubled size of a study, funding is often limiting studies of gender differences.

WP C:7 Ethics

Summary description of WP objectives
i) To implement a system for monitoring of ethical issues within the PHIME project;
ii) To organise a workshop on this system on the kick-off meeting;
iii) To implement recommendations of the Ethical review.

Contractors involved
UUC (WP leader Julie Wallace), All Partners in WP I-III.

Degree to which the objectives were reached
All achieved.

Methodologies and approaches employed
A monitoring system was put in place to check receipt of ethical approval by Partners for human studies. Ethical reviews of the different studies within PHIME were monitored by the Partner in charge of this WP (UUC). Each Partner on receipt of approval for a study was required to send confirmation to the WP leader and copy the information to the PHIME Co-ordinator.

A workshop was organised at the PHIME kick-off meeting to provide information to Partners and allow discussion on potential ethical issues.

Achievements in relation to the state-of-the-art
Not relevant.

Conclusions
Highlighting ethics as a specific WP in PHIME has put additional safeguards in place to ensure that all research involving human volunteers within PHIME is undertaken according to national ethical requirements.

WP C:8 Intellectual Property Rights

Summary description of WP objectives
- To report IPR issues to the Project Management Committee and to support partners in seeking of patents;
- To ensure the protection and optimal exploitation of commercially valuable results.

Contractors involved
York (WP leader Dale Sander); service aimed at all Partners.

Degree to which the objectives were reached
Achieved, in the sense that there were resources, both in knowledge and funding, to identify IPR issues and support Partners in seeking assistance or patents.
The project resulted in one patent in the area of environmental analyses. However, the partner responsible did not seek support from the IPR workpackage.

Methodologies and approaches employed
Partner York has, on a yearly basis, informed all project Partners of the resources and knowledge available for IPR-related activities. Also, the Project Executive Committee has received bi-yearly updates.

Achievements in relation to the state-of-the-art
Not relevant.

Conclusions
At the outset of the Project, it was expected that IPR issues may arise, especially within Pillar IV, and possibly as regarded genes involved in disease. Although Partners were repeatedly reminded of the funding and know how available to them, the partner responsible for the patent application did not seek support from the IPR workpackage.

WP C:9 Dissemination

Summary description of WP objectives
1. Dissemination of Project results to the EC services, international and national agencies, mass media, food producers and consumers, apart from the scientific community;
2. Keeping Partners fully informed about Project-related issues, in order to obtain maximum transparency and synergy;
3. Internal home page for Project internal communication;
4. Logo and colour brochure;
5. External webpage, open to the public;

6. Cooperation/communication with other EC-funded projects;
7. Info-packs of less than one page: a) for journalists and decision-makers, b) popular version;
8. Production of press releases;
9. Organisation of workshops;
10. Encouragement of PHIME scientists in participating in conferences, symposia workshops etc.;
11. Disseminating the rules of publishing to the Project Partners.

Contractors involved
LU (WP leader Lina Löfmark), York, All Partners.

Degree to which the objectives were reached
All planned fields of dissemination have been treated in the WP. However, varying emphasis has been placed on the different aspects of communication.

Methodologies and approaches employed
A communication strategy was adopted by the Project Executive Committee (PEC) at the beginning of the Project. This served as a guide for the WP effort. In the fourth reporting period, this plan was supplemented with a Plan on final dissemination.

All Partners were encouraged to disseminate information on their work as broadly as possible. As many Project Partners are accustomed to this, and have the necessary networks - locally, regionally, nationally and internationally - a lot of the communication work was not coordinated by the Project Office. On the local and regional, and sometimes national levels, the media exposure of some WPs was substantial.

The Project Office has gained a lot of knowledge, ideas and contacts through participation in the communication network COMMNET.

Ad 1, 9. PHIME has disseminated its results through workshops at EEA and ECHA. EFSA staff has participated in these events, as well as at a PHIME seminar aimed at Commission staff in Brussels. PHIME has arranged seminars for national agencies and policymakers. Other international agencies have been invited to the meetings arranged.

The project has sought cooperation with food producers, who have, together with consumer organisations, been invited to the public part of the final meeting in Brussels in February 2011. PHIME was one of the organisers of European Food Science Day in Brussels in November 2009.

The PHIME newsletter has been produced two to four times yearly and has contained updates of partial results and methodological issues of interest to a wider audience. The newsletter has been distributed through a mailing list and posted on the project home page.

Ad 2, 3. The internal communication within the Project has run very smoothly and efficiently. The atmosphere within the Project has been good, fully in line with the coordinator and Project Office's ambitions, as this is a prerequisite for successful cooperation.

The Project Office has mainly communicated through e-mail and telephone. Contacts between Partners have followed the same pattern. The Project Office has also kept the Consortium updated on issues of common interest and concern through "Status reports" every few months.

In order to create and intensify cooperation between Pillars, the sharing of information between Pillars has been on the agenda of all PEC meetings. Also, the Project has arranged workshops at Annual Meetings, with the aim of creating an interest in cross-Pillar cooperation.

The internal home page has mainly been used for the posting of key documents and templates although other functions were built into it. Partners have clearly not felt the need to use these.

Ad 4. The PHIME brochure has been reprinted once.

Ad 5. The external webpage has gone through several changes, and thus required a lot of work. In the end, the page was created with an open source content management system. However, this system still needed a lot of technical support. It also cost a lot more than is expected by the EC, even though we chose a "budget option". The number of non-PHIME visitors to the page has been limited, likely because key results are only available at the end of the Project.

PHIME set up a Facebook page towards the end of the project. For the younger researchers within the Project, this communication path may have been of value, but few external persons have followed the page.

Ad 6. PHIME has been persistent in seeking cooperation with other EU-funded projects and has been the initiating party in all its cases. This has led to some valuable common activities, notably the exchange of samples with NewGeneris, which resulted in a common manuscript, and a joint conference with NoMiracle. PHIME has invited other projects to Annuals Meetings (NewGeneris, INTARESE, EnviroGenomarkers, ECNIS), training activities and other appropriate events. Surprisingly, however, some key projects (notably ESBIO, DEMOCOPHES and COPHES) have showed limited interest in cooperation. The Coordinator has lectured at a meeting in NoMiracle, the Deptuty Coordinator in COPHES.

Ad 7. PHIME is preparing information sheets on key findings of interest for the society. They are being distributed to science journalists by Partners. The Commission communications department is also expected to help with this.

Ad 8. Centrally produced press releases with a local twist, concerning PHIME work have been issued for Annual Meetings, with limited success. Press conferences were arranged for the kickoff and two first Annual Meetings, but then replaced by contacts with selected media. This worked reasonably well where the hosting scientists had already well established media connections. Some Partners have been very efficient in attracting media attention, on local, regional, national and sometimes international level.

Ad 10. Partners have generally been very active in participating in conferences, etc. PEC has granted support from the training budget to all requests put forward.

Ad 11. The rules of publishing have been posted on the internal home page. All Partners have been reminded of the rules at the Annual Meetings.

The PEC and the Project Coordination Committee (PCC; merged in Yr 2, after first review) held the major part of their meetings in the form of video conferences. This saved a lot of time and money for the researchers, apart from being environmentally friendly. This meeting form generally worked well, but the technical setup was a reoccurring issue. In order to make this work, Members of the two bodies had first met in person and got to know each other in the initial phase.

Several PHIME scientists have been members of of EFSA bodies during the project time (Sean Strain, Agneta Åkesson, Philippe Grandjean). The Coordinator is a former member of the EFSA Scientific Committee, and also has close links to the EEA. Thereby, PHIME results have been fed into the system. PHIME has also arranged seminars at EEA, ECHA and DGResearch directed at EC staff.

Conclusions
Information on epidemiological studies (such as in Pillars I-III) is, outside the scientific community, best communicated only when the results are obtained. As this happens only at the end of the Project, when the money and time is up, there are limited possibilities to communicate with audiences outside the readers of scientific articles. It would be appropriate to grant this type of projects 6 months of dissemination work, with funding specifically for this, at the end of the project time. In experimental work (Pillar IV), the production of interesting results is a more continuous process.

For pure research projects, like PHIME, there is the risk of wearing out the stake holders before any results can be presented, if you bombard them with information during the project period. Of course, a certain interest has to be created, but without results you risk loosing the interest by the time when vasluable results are actually presented. This causes a delay in the absorption of the knowledge into the society, as it has to go through the loop of publication before perculating into the decision-making process.

A Project Office cannot communicate efficiently in Partners' countries, although it is fit to lead the communication effort and offer support and advice to Partners. Therefore, communication at national, regional and local levels is dependent on partners' media contacts and interest in communication.

The Project has noted a lack of interest and/or capacity in the European Community to rapidly absorb and make use of the results and advise of the Project. With the resources spent on financing the research of the Project, both financial and in the form of evaluation and financial control, this is remarkable.

We recommend the establishment of an elaborate home page (internal or external) only were the need is expressed by the scientists. Keeping the home page updated, trying to get scientists to update it, and the high costs, are reasons to keep the home page to the level of real need. The Project Office considers the EC calculations for home pages to be grossly understated. Both pre-existing platforms and open-source adapted ones are expensive and time consuming.

WP 0 Management

Below, we list managerial issues and problems which may be of help to future projects.

Management structure
PHIME's management structure was based on two bodies:

- Project Coordination Committee (PCC), consisting of the Pillar leaders and the Coordinator - five Members. Responsibility for the management of the project;
- Project Executive Committee (PEC), consisting of the PCC members and the WP leaders for WP C:1 (QC/QA), C:7 (Ethics), and C:8 (IPR) – eight Members. This group was expanded during the project time, as the two Pillar II sub-pillar leaders were included. Responsibility for the scientific activities and integration of the project;

At the first revision, the need of two separate bodies was questioned. The Project then decided to let the two bodies meet in concert, as they overlapped for the greatest part. Only few cases occurred where only the PCC members had the right of vote. We therefore recommend future projects to keep the management structure as simple as possible.

Scientific Advisory Board
The body consisted of five senior scientists. The input and advice of the experts in this group was of utmost importance to the Project. They were especially helpful at the outset, when the different WPs elaborated their methods and study protocols. However, their support and good advice was an asset during the whole Project, and their input was important for the integration between Pillars. They were active in the WP C:4 workshops, which created the interest in exploring common fields of interest. We recommend choosing board members with great expertise, but also good social skills.

Video conferences
PCC/PEC meetings, as well as WP discussions, have to a large extent been held in the form of video conferences. Apart from the technical issues, this has worked very well. It has saved a lot of time and money, apart from the environmental gains. If all partners are equipped with cameras and microphones in their offices, this is an even more efficient option. However, seeing each other is important, as it creates lively and focused discussions.

Defaulting parties
PHIME had a problem with a defaulting Partner. However, this was brought to the EC Scientific Officer's attention only in the fourth year. Had this been made clear earlier, not least in the yearly reporting, the defaulting party could have been replaced. We therefore recommend early and serious discussion with the defaulting party, as well as with the Scientific Officer.

Targeted Third Country members
During the first year of the Project, PHIME submitted a proposal to the "INCO extension call" to add three TTC members. PHIME partners were asked to propose candidates which fulfilled the following criteria, set by the General Assembly. It is quite likely that the following criteria/possibilities laid a good foundation for successful cooperation once the new partners entered the project:

- Manageable;
- Fits into the Project/WPs

- Mixed exposure;
- Enlarged cohorts;
- Gene-environment interaction;
- Ongoing cooperation with a Partner;
- Analytical capacity;
- Possibility to measure outcome (disease);
- Excellence, own and contribution to PHIME's.

Section 2 Dissemination and use

Publishable Results: Publications in peer-reviewed journals

2006

Arrivault S, Senger T, Krämer U (2006) The Arabidopsis metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. Plant Journal 46: 861-879
Clemens, S. (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie 88: 1707-1719
GIBIČAR, Darija, HORVAT, Milena, NAKOU, Sheena, SARAFIDOU, Jasmin, YAGER, Janice W. Pilot study of intrauterine exposure to methylmercury in Eastern Aegean islands, Greece. Sci. total environ.. [Print ed.], 2006, vol. 367, str. 586-595.
Kobal Grum, D, Kobal, A B, Arneric, N, Horvat, M, Zenko, B, Dzeroski, S, Osredkar, J. Environmental Health Perspectives 2006, Vol 114, no 1 "Personality Traits in Miners with Past Occupational Elemental Mercury Exposure"
Suwasono Y, Sand S, Vahter M, Falk Filipsson A, Skerfving S, Lidfeldt J, Åkesson A. Benchmark dose for cadmium-induced renal effects in humans. Environ Health Perspect 2006;7:1072-1076.
Yager, Janice W., Horvat, Milena. Recent findings in mercury health effects. Environ. health perspect., 2006, vol. 114, str. 289.
Åkesson A, Bjellerup P, Lundh T, Lidfeldt J, Nerbrand C, Samsioe G, Skerfving S, Vahter M. Cadmium-induced effects on bone in a population-based study of women. Environ Health Perspect 2006;6:830-834.

2007

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