UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

SELECTION AND COMBINATION OF BIOMARKERS TO CONTROL AND PREVENT THE RISK OF OCCUPATIONAL EXPOSURE TO THE MIXTURE OF LEAD, ARSENIC AND MANGANESE

Vanda Maria Falcão Espada Lopes de Andrade

DOUTORAMENTO EM FARMÁCIA

(especialidade de TOXICOLOGIA)

2014

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Tese orientada pela Professora Doutora Ana Paula Marreilha dos Santos, Professora Auxiliar da Faculdade de Farmácia da Universidade de Lisboa e co-orientada pela Professora Doutora Maria Camila Canteiro Batoréu, Professora Associada com Agregação à Faculdade de Farmácia da Universidade de Lisboa, elaborada para a obtenção do grau de Doutor em FARMÁCIA, especialidade Toxicologia.

2014

Acknowledgments

I would like to express my gratitude to my supervisor Profª Ana Paula Marreilha dos Santos for her vast knowledge and experience always joined with simplicity, her calm and patience specially during the times when these skills were definitely lacking in my person, for the huge amount of cookies and chocolates eaten while reviewing papers with her and most of all for her friendship.

I also would like to thank to my co-supervisor Profª Maria Camila Batoréu for making me a Toxicology fan few minutes after giving my first Toxicology lecture, the transmission of her incredible knowledge, subtle sense of humor, sincerity, constant encouragement and friendship.

I am thankful to Profª Maria Luisa Mateus for the interested guidance and support given in the development of some of the analytical methodologies used during this work. I also thank for her patience and constant good mood.

I am grateful to Prof. Michael Aschner from Albert Einstein College, USA for his advices during a part of my work.

I thank Profª Maria Eduarda Mendes for her sympathy, help and instructions that were very useful for some of the metal analysis performed during the development of some experimental work concerning the atomic absorption.

I wish to thank to Helena Brito and Verónica Silva for all their laboratory support, the help provided during the in vivo assay and their companionship.

I thank to Dr. Edgar Almeida for all his support and collaboration concerning the offer of the urine and blood samples from the mining company.

I thank to Dra. Elisa Alves and Prof. Leonor Correia for the offer of the urine and blood samples from non-exposed humans.

I also thank to my colleagues Edite Torres, Dinamene Santos, Vasco Branco, Neusa Figueiredo and Inês Vaz for their friendship, support and for the excellent working environment.

I thank to my companion Rui and to my mother for the support and comprehension during these years.

Abstract

Lead (Pb), arsenic (As) and manganese (Mn) are metals with neurotoxic effects that are present in mines as metal mixtures, making miners potentially vulnerable to an increased risk of neurotoxicity. Biomarkers can be crucial tools to detect these disorders in their earlier stages enabling patients to avoid the disease progression. Several biomarkers for Pb, As and Mn have been investigated, yet only for single exposures despite the different metals can interact within the brain. The aim of this work was to identify biomarkers of neurotoxicity which may be used to control and prevent the risk of chronic low levels exposure to the mixture of Pb, As and Mn. After performing an in vivo assay with Wistar rats repeatedly exposed (for 8 days) to Pb, As, Mn or the 3-metal mixture, the co-exposure increased behavioral toxicity and augmented Pb's deposition in brain more than upon single exposures, with blood Pb levels failing to reflect this alteration, thus, contributing to underestimate the risk associated with Pb-induced damages. Mechanistic insights concerning the consequences of in vivo Pb, As and Mn interactions revealed changes in neurotransmitters and heme precursors' levels in the brain. Changes of these BMs in peripheral samples suggested their suitability to be used as BMs of exposure and/or neurotoxicity upon exposure to this mixture. A multibiomarker approach based on multiparameter analysis of independent markers was used; the results suggest that the combination of biomarkers is a better tool to predict the type of exposures and the magnitude of neurotoxic effects, better than when used alone. A preliminary human study in a mining population was performed to apply the results obtained in the in vivo model. According to the results, the miners might be exposed to excessive levels of Pb, As and Mn, and the integration of experimentally selected BMs, Pb and Mn blood levels and urinary Pb, As and delta-aminolevulinic acid levels, revealed to be a good tool to identify excessive exposed workers. Exploration of BMs' integration methodologies seems certainly promising to assess "real-life" scenarios of exposure to chemical mixtures.

Keywords

Lead, arsenic, manganese, mixtures, biomarkers, integration, neurotoxicity

Resumo

Introdução: O aumento crescente da exposição humana a metais é atualmente um problema de Saúde Pública. De entre os diversos metais, o chumbo (Pb) e o arsénio (As) são dos principais metais detetados em diversos contextos ambientais, e no que se refere ao Mn, as suas emissões são também significativas com efeitos nefastos para a saúde pública dado a sua vasta utilização na indústria metalúrgica. Os três metais têm efeitos neurotóxicos e estão presentes na forma de misturas em diversos locais de trabalho, como nas minas, resultando para os mineiros um risco acrescido de neurotoxicidade. A exposição a metais com efeitos neurotóxicos leva frequentemente a desordens neurológicas crónicas, progressivas, sem sinais de alerta, e quando os sintomas são clinicamente percetíveis, os efeitos podem ser muitas vezes irreversíveis. É neste contexto que os biomarcadores podem constituir ferramentas cruciais para a deteção de exposições excessivas a metais e assinalar atempadamente o aparecimento de efeitos adversos na fase inicial, enquanto o efeito tóxico for ainda reversível. Diversos biomarcadores têm vindo a ser propostos para avaliação da exposição ao Pb, ao As e ao Mn, contudo apenas para exposições a cada um dos metais isoladamente. Embora estudos de mecanismos de interação de metais no cérebro sejam conhecidos, há ainda muito trabalho a desenvolver no que respeita à pesquisa de biomarcadores de neurotoxicidade induzida pela exposição a misturas de metais. Nesta perspetiva, a Toxicologia Preditiva é uma área que utiliza ferramentas estatísticas para integrar os resultados experimentais de modelos toxicológicos (in vitro e in vivo) e epidemiológicos. Esta área tem vindo a revelar-se promissora na deteção precoce de diversas doenças, nomeadamente as neurodegenerativas integrando resultados de painéis de biomarcadores de exposição e efeito. Pretendeu-se com este trabalho identificar biomarcadores de neurotoxicidade para o controlo e prevenção do risco de exposição crónica à mistura de Pb, As e Mn. Os objetivos específicos consistiram em: i- avaliar efeitos neurotóxicos induzidos por esta mistura num modelo in vivo; ii- estudar o mecanismo das interações entre o Pb, o As e o Mn na sua disposição em órgãos alvo; iii- identificar a co-exposição e estimar a severidade dos efeitos neurotóxicos através

de procedimentos preditivos, mediante a integração de biomarcadores recorrendo a ferramentas estatísticas; e iv- fazer a translação dos resultados experimentais obtidos no estudo in vivo para um estudo preliminar realizado numa população de mineiros co-expostos aos mesmos metais. **Metodologia:** foi realizado um ensaio de exposição repetida com cinco grupos de ratos Wistar a que foram administradas 8 doses de, Pb (5 mg/Kg/dia), As (60 mg/L) e Mn (10 mg/Kg/dia), sendo nos Grupos I, II e III administrado cada metal isoladamente, no Grupo IV, administrada a mistura dos metais, e tendo constituído o Grupo V, o grupo controlo. A atividade motora foi avaliada no início da experiência e 24 horas após a administração da última dose, assim como a recolha de urina de 24h. No final do ensaio e após o sacrifício dos ratos procedeu-se à recolha do sangue, cérebro, rim e fígado para determinação dos seguintes biomarcadores: níveis dos 3 metais em todas as amostras biológicas, atividade da acetilcolinesterase no cérebro e no sangue, níveis de ácido delta-aminolevulínico e o perfil de porfirinas no cérebro e urina, prolactina no soro e porfirinas totais na urina. Os resultados obtidos foram utilizados para selecionar biomarcadores periféricos de exposição e efeito considerando que: 1) os melhores biomarcadores de exposição permitiam distinguir de entre os ratos expostos a cada metal, ratos expostos à mistura dos 3 metais, e ratos controlo; 2) e os melhores biomarcadores de efeito apresentavam uma boa correlação com os resultados obtidos nos ensaios de atividade motora. Os biomarcadores selecionados em 1) e 2) foram posteriormente integrados, em procedimentos preditivos recorrendo a ferramentas estatísticas. Aplicaram-se 2 procedimentos preditivos: o primeiro combinando os níveis dos 3 metais no sangue e/ou urina, as concentrações urinárias de ácido delta-aminolevulínico e das porfirinas totais, a atividade da acetilcolinesterase no sangue e a prolactina no soro; e o segundo, integrando o perfil urinário das porfirinas, uma alternativa mais prática e menos dispendiosa. Cada um dos procedimentos consistiu numa 1ª fase que recorrendo a análise discriminante, visou identificar o tipo de exposição de cada rato, e numa 2ª fase, que através de análise de regressão multivariada se destinou a estimar a magnitude dos efeitos neurotóxicos. Através de um estudo preliminar, os 2 procedimentos preditivos foram testados posteriormente numa população de mineiros de uma mina no Alentejo em que os trabalhadores estavam co-expostos ao Pb, As e Mn. Neste estudo preliminar foram utilizadas 2

populações controlo: um grupo de indivíduos de uma área urbana (Lisboa) e um grupo constituído pelos trabalhadores administrativos da empresa mineira que vivem numa zona rural. **Resultados:** os ratos tratados com a mistura de Pb, As e Mn diminuíram significativamente ($p<0,05$) a atividade motora relativamente aos restantes grupos, exceto relativamente à atividade vertical quando comparada com o grupo exposto ao Pb. Foram detetadas interações significativas ($p<0,05$) entre os três metais ao nível do cérebro. Neste órgão, os níveis de Pb e ácido delta-aminolevulínico revelaram-se mais elevados ($p<0,05$) quando comparados com os ratos tratados com apenas um dos metais, e a atividade da acetilcolinesterase diminuiu significativamente ($p<0,05$), quando comparada com os controlos e com os ratos tratados unicamente com As. Apesar de não relevantes, detetaram-se também alterações no perfil de porfirinas no cérebro. No que diz respeito aos níveis de metais no sangue, não foram observadas diferenças significativas entre o grupo tratado com a mistura e os grupos tratados com apenas um dos metais, e não foram igualmente detetadas diferenças na atividade da acetilcolinesterase entre os 5 grupos. As concentrações de prolactina no soro de ratos co-expostos revelaram-se significativamente ($p<0,05$) mais elevadas que nos controlos, assim como a excreção urinária de Pb, Mn, ácido delta-aminolevulínico e porfirinas totais quando comparadas com todos os restantes grupos ($p<0,05$). Além disso, as alterações destes biomarcadores observadas em amostras periféricas foram sugestivas da sua aplicabilidade como indicadores da co-exposição e de efeitos neurotóxicos. Contudo nenhum biomarcador isoladamente se revelou ser suficiente para identificar o tipo de exposição e/ou prever a intensidade dos efeitos neurotóxicos. Desta forma optou-se por combinar os biomarcadores recorrendo a ferramentas estatísticas. A integração de biomarcadores no primeiro procedimento permitiu identificar corretamente o tipo de tratamento administrado a cada rato em 96,7% dos casos. Por sua vez, a atividade motora dos ratos co-expostos foi estimada pelo modelo com um erro de 11% na atividade horizontal (relativo ao número médio de movimentos dos ratos controlo) e de 13% na atividade vertical. A integração do perfil urinário das porfirinas levou à classificação correta do tipo de exposição em 90% dos ratos e teve a capacidade de estimar a severidade das desordens motoras com erros de 11 e 16%, para as atividades horizontal e vertical, respetivamente. Quanto aos resultados obtidos no estudo preliminar realizado nos trabalhadores das minas, estes trabalhadores

apresentaram os níveis dos 3 metais aumentados no sangue, embora tratando-se de uma alteração ligeira e sem significado estatístico relativamente aos trabalhadores administrativos (controlo). A excreção urinária de Pb, As e Mn revelou-se mais elevada que em ambos os grupos controlo, embora as diferenças observadas nos níveis de Pb não tivessem significado estatístico. A atividade da acetilcolinesterase no sangue e a excreção de porfirinas totais revelou-se significativamente mais elevada, ao comparar estes valores com os dos trabalhadores administrativos. A aplicação de procedimentos preditivos obtidos experimentalmente conduziu à identificação correta de 97,1% de todos os indivíduos quanto ao seu tipo de exposição. Por sua vez a integração do perfil urinário de porfirinas classificou corretamente 61,7% dos casos. **Conclusões:** Este trabalho evidenciou que a co-exposição ao Pb, As e Mn através de um modelo experimental aumentou significativamente a toxicidade a nível da atividade motora, quando comparada com a exposição a cada um dos metais separadamente. O tratamento com a mistura levou também ao aumento da acumulação de Pb no cérebro e no rim, relativamente à exposição ao Pb isoladamente. Além disso, verificou-se que os níveis de Pb no sangue de ratos co-expostos não refletiram a acumulação acrescida de Pb no cérebro, o que pode implicar que o risco de neurotoxicidade induzido pela exposição à mistura de Pb, As e Mn seja subestimado através da monitorização da exposição através do doseamento de Pb no sangue. A co-exposição a estes metais revelou pela primeira vez consequências da interação destes elementos ao nível de neurotransmissores e à acumulação de precursores da síntese do heme no cérebro, que quando em excesso podem atuar como toxinas. As alterações destes biomarcadores observadas em amostras periféricas foram sugestivas da sua aplicabilidade como indicadores da co-exposição e de efeitos neurotóxicos, mas nenhum revelou ser específico do tipo de exposição quando aplicado isoladamente. Desta forma a integração dos biomarcadores selecionados revelou-se a melhor ferramenta preditiva, tanto em ratos como no estudo em humanos no que concerne à identificação da exposição. No que se refere á seleção de biomarcadores preditivos de neurotoxicidade e estimativa da magnitude dos efeitos neurotóxicos, esta só foi obtida no estudo em ratos, mas mais estudos em humanos estão previstos a curto termo. A combinação de biomarcadores de exposição e efeito no controlo da exposição a

misturas de metais é uma nova abordagem que poderá contribuir para a prevenção do risco de neurotoxicidade nos contextos ocupacionais abordados.

Palavras-chave

Chumbo, arsénio, manganês, misturas, biomarcadores, integração, neurotoxicidade

General Index

Initial Statement

Parts of this thesis have been published previously in international peer-reviewed journals:

Andrade, V., Mateus, M.L., Batoréu, M. C., Aschner, M. and Marreilha dos Santos, A.P. (2014). Changes in rat urinary porphyrin profiles predict the magnitude of the neurotoxic effects induced by a mixture of lead, arsenic and manganese (submitted to Neurotoxicology).

Andrade, V., Mateus, M. L., Santos, D., Aschner, M., Batoréu, M.C. and Marreilha dos Santos, A. P. (2014). Arsenic and Manganese Alter Lead Deposition in the Rat. Biol Trace Elem Res. 158(3), 384-391.

Andrade, V, Mateus, ML, Batoréu, MC, Aschner, M and Marreilha dos Santos, AP. (2013). Urinary delta-ALA: A potential biomarker of exposure and neurotoxic effect in rats co-treated with a mixture of lead, arsenic and manganese. Neurotoxicology. 11 (38), 33-41.

Some results were also presented in scientific meetings:

<u>Communications in poster in international congresses:</u>

Mateus, L., Andrade, V., Catarina, F., Batoréu, C. and Marreilha dos Santos, A.P. (2012). Urinary Porphyrins as Biomarkers of Neurotoxicity in Rats Exposed to a Mixture of Metals. "48th EUROTOX Congress, June 2012, Sweden".

Marreilha dos Santos, A.P., Andrade, V., Mateus, M.L., Aschner, M. and Batoréu M.C. (2010). Occupational exposure of miners to manganese and lead – A preliminary study. "XII International Congress of Toxicology (IUTOX 2010)", Barcelona, 11-15 July; Abstracts/Toxicology Letters 2010; 196 (17): S79.

Communications in poster in national congresses:

Andrade, V., Mateus, M.L., Batoréu, M.C., Aschner, M. and Marreilha dos Santos, A.P. (2013). Urinary delta-ALA as a potential biomarker of neurotoxicity in rats co-exposed to a mixture of metals. 5th iMed.UL Postgraduate Students Meeting, iMed.UL/Chemical Biology and Toxicology", Faculty of Pharmacy, University of Lisbon.

Andrade, V., Batoréu, M.C. and Marreilha dos Santos, A.P. (2011). Changes on the Toxicokinetics of Arsenic, Manganese and Lead in Rats Co-exposed to the Mixture of These Metals. "3rd iMed.UL Post-Graduate Students Meeting", iMed.UL/Chemical Biology and Toxicology", Faculty of Pharmacy, University of Lisbon.

Oral communications in international congresses:

Andrade, V. (2013). Evaluation of central nervous system parameters underlying mechanisms of arsenic (III)-induced neurotoxicity. International Conference on Occupational & Environmental Toxicology (ICOETox 2013), Porto (Portugal).

Oral communications in national congresses:

Andrade, V. (2013). Interference of arsenic and manganese in lead accumulation and excretion in rats exposed to a mixture of these metals. 6th Postgraduate iMed.UL Students Meeting, Faculty of Pharmacy, University of Lisbon.

List of abbreviations

ACGIH	American Conference of Governmental Industrial Hygienists
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer disease
ADME	Absorption, distribution, metabolism and excretion
ALA	Aminolevulinic acid
ALAD	Aminolevulinic acid dehydratase
ALAS	Aminolevulinic acid synthase
ASTDR	Agency for Toxic Substances and Disease Registry
B	Blood
BBB	Blood brain barrier
BChE	Butyrylcholinesterase
BEI	Biological exposure index
BLV	Biological limit values
BM	Biomarker
Br	Brain
BW	Body weight
CAT	Catalase
CF	Classification functions
CNS	Central Nervous System
COX	Cyclooxigenase
Creat	Creatinine
D1	Dopamine receptor 1
D2	Dopamine receptor 2
DA	Dopamine
DAT	Dopamine transporter
DMA	Dimethylarsenic

DMT1	Divalent metal transporter 1
EPA	Environmental Protection Agency
FECH	Ferrochelatase
GABA	Gamma-aminobutyric acid
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
GI	Gastrointestinal
GLUT1	Glucose transporter 1
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
H.I.	Hazard index
Hb-Fe(II)	Oxyhemoglobin
HGAAS	Hydride generation atomic absorption spectrometry
HMB	Hydroxymethylbillane
HMBS	Hydroxymethylbillane synthase
HPLC	High performance liquid chromatography
ICOH	International Commission on Occupational Health
Ip	Intraperitoneal
IQ	Intelligence quotient
JSOH	Japan Society for Occupational Health
LC-ICPMS	Liquid chromatography inductively coupled plasma mass spectrometry
L-DOPA	Levodopa
LMND	Lower motor neuron disease
MAK	*Maximale Arbeitsplatz-Konzentration*
MAO	Monoamine oxidase
MAP	Mitogene-activated protein
MDA	Malonilaldehyde
MMA	Monomethylarsenic

MMT	Methylcyclopentadienyl manganese tricarbonyl
Mn-SOD	Manganese superoxide dismutase
MT	Metallothioneins
NIOSH	National Institute for Occupational Safety and Health
NOS	Nitric oxide synthase
OELs	Occupational exposure limits
OS	Oxidative stress
OSHA	Occupational Safety & Health Administration
PBG	Porphobilinogen
PD	Parkinson disease
PEL	Permissible exposure limit
PNS	Peripheral nervous system
PRL	Prolactin
Porf T	Total porphyrins
QL	Quantification limit
REL	Recommended exposure limit
RBC	Red blood cells
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
SCOEL	Scientific Committee on Occupational Exposure Limits
TH	Tyrosine hydroxylase
TIDA	Tuberoinfundibular dopamine
TLV	Threshold Limit Values
TWA	Time-weighted average
U	Urine
VIF	Variance Inflation Factors
VWA	Victorian Workcover Authority
WHO	World Health Organization

List of chemical formulas

•OH	Hydroxyl radical
$1O_2$	Singlet oxygen
ALA•	Aminolevulinic acid-enoyl radical
$(CH_3)_2As$•	Dimethylarsinic radical
$(CH_3)2AsOO$•	Dimethylarsinic peroxyl radical
H_2O_2	Hydrogen peroxide
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2^-•	Superoxide anion radical
ONOO−	Peroxynitrite
SH	Sulfhydryl

Chapter 1

General introduction

1.1. Metals in the environment

Pollution is a world problem with immeasurable consequences and toxic metals are compounds frequently found as their components (Rodrigues et al, 1996) because their intensive use by modern society has, over the years, leaded to an increase of their levels in the biosphere (Al-Attar, 2011; Kakkar and Jaffery, 2005). Elevated concentrations of metals in the environment as a result of human activities have been recorded since ancient times (Wong et al, 2006), particularly since the middle of the 19th century, when the production of heavy metals increased steeply for more than 100 years. At the end of the 20th century, the emissions of heavy metals started to decrease in developed countries, however in less developed regions is even increasing (Järup, 2003). As generally considered, there are no frontiers for pollution and therefore, globally the contamination of the environment with heavy metals continues to grow. Furthermore, industrial metallic emissions into the atmosphere can have a short life-time (few days), but they travel long distances from their point of emission, accumulate on land and form sediments in lakes and rivers, where their lifetime can reach from a few hundred years to several thousand years (Rodríguez et al, 1998). Unlike organic chemicals, metals are neither created nor destroyed by biological or chemical processes (Wong et al, 2006) and consequently they can accrue in the environment, passing up to the food chain and accumulate in the tissues of living organisms (Al-Attar, 2011). For these reasons, metals exposures to general population, through air, food or drinking water has become an increasing and a global phenomenon (Martinez- Finley et al, 2012; Wong et al, 2006).

1.1.1. Environmental exposure to metal mixtures

Along with actual apprehensions pertaining to human exposures to metals, it is becoming recognized that environmental exposures are not to single chemicals. The truth is that exposure to mixtures is the environmental reality in a nowadays chemically sophisticated world (Simmons, 1995). All environmental media have naturally occurring mixtures of

metals and metals are often introduced into the environment as mixtures (Fairbrother et al, 2007). These mixtures are ubiquitous in air, water and soil (Simmons, 1995) and thus, people are exposed either concurrently or sequentially, by various routes of exposure and from a variety of sources, to a large numbers of toxicants at low doses that may result in similar or dissimilar effects over exposure periods that can range from short-term to a lifetime (ASTDR, 2000, Pohl et al, 1997). In this perspective, and according with the public concern, the U.S. Environmental Protection Agency (EPA) recommends greater efforts on the understanding of the combined toxic effects of metals (ASTDR, 2000; Fairbrother et al, 2007), although the undergone studies have been largely concerned with single exposures (Kortenkamp and Faust, 2009; Pohl et al, 1997). To illustrate, in 1994, 94.3% of 122 technical reports from the National Toxicology Program described the chronic toxicity of single chemicals (Simmons, 1995) and still in 2006, most of the performed studies focused on the adverse health effects of toxic metals were conducted on populations with relatively high exposure and the majority considered individual metals (Kossowska et al, 2013). Until when mixtures are viewed as a simple collection of a few component chemicals, the poor understanding of the nature and the magnitude of toxicological interactions will lead to great uncertainties (ASTDR, 2000) and the practical consequence of the strategy of studying single metal exposures may be the fail, underestimating the health risks of affected populations (Kordas et al, 2010; Kortenkamp and Faust, 2009). Further, we are defied to assess the potential health risk of multiple exposures, and gradually substitute (unrealistic) single compound-oriented standard setting for mixture-oriented (real life-oriented) standard setting (Feron et al, 1995).

Fortunately studies of chemical mixtures are starting to progress by incorporating more knowledge of specific modes of toxicological action and greater use of statistical methods and mathematical models (ASTDR, 2000). Even so, predicting the health consequences of multiple chemical exposures is still a challenge (Pohl et al, 1997) because their study incorporates the understanding of interactions at several levels occurring: i) in the environmental media that may modulate the external exposure (external dose), ii) during processes of speciation, binding and transport; iii) at site(s) of uptake and/or elimination

from the organisms, resulting in modifications of the total accumulated internal dose; iv) and at the target site may affecting the binding of one of more chemicals to a receptor through which toxicity may be mediated. These interactions may change toxicokinetics and toxicodynamics (Spurgeon et al, 2010). That way mixtures can influence the expected adverse health effects and this is particularly worrying when the components of the mixture individually attack the same organs or, together, overwhelm a particular mechanism essential for the organism to defend itself against toxic substances (Calderon et al, 2003), eventually resulting in greater (synergistic) toxicity (Lister et al, 2011). Low doses that might not individually cause health effects, in concert may become a public health issue (Calderon et al 2003). It is reported that exposure to metal mixtures at concentrations below environmental quality guideline levels for individual components resulted in adverse effects, that were attributed to interactions among the components (Yen Le et al, 2013). This matter was recently recognized by the U.S. EPA as a key gap in metal risk assessments (Abboud and Wilkinson, 2013); thus, a demand exists for the research into the toxicity of metals, very specially as regards to metal mixtures in trace levels (Kim et al, 2009).

1.1.2. Criteria to select metal mixtures

Given the almost infinite number of chemical mixtures, regulators are faced with the problem of the choice of chemicals to joint assessment and regulation (ASTDR, 2004; Kortenkamp and Faust, 2009). Components produced and emitted together from an industrial process or that occur together in the same environment or human body compartment are certainly to be considered (despite there are still considerable knowledge gaps concerning to mixture of chemicals present in human tissues). Chemicals thought to exhibit their effects through common mechanisms have been often grouped together based on similarities in chemical structure or derived from mechanistic considerations. Recently it has been argued that grouping criteria should focus on common adverse outcomes, with less emphasis on similarity of mechanisms (Kortenkamp

and Faust, 2009) and that attention should be focused on those having the greatest potential impact on human health (ASTDR, 2004). Also, for practical purposes, criteria are needed to define the relevant components of a mixture. Such criteria cannot rely simply on the concentrations of the compounds in the mixture, but must also take note of the expected contribution to relevant endpoints of toxicity (Kortenkamp and Faust, 2009). Reproductive, carcinogenic and neurotoxic effects are considered potentially important health end points in epidemiological studies of complex mixtures, most particularly when such mixtures contain trace metals (Shy, 1993). Exposure to neurotoxic agents represents indeed a concern of high priority in modern society, given the constantly increasing reported frequency of neurological diseases (Lucchini and Zimmerman, 2009), where apprehension has been also growing with respect to the induced long-term effects (Emerit et al, 2004).

Metals have several particularities that should be taken in account when accessing the risks of their exposure: metals can be transformed by biological or chemical processes into species with different valence states and be converted between inorganic and organic forms. All these forms may present different behaviors, such as absorption, distribution, transformation, and excretion and/or different toxicities; metals do not require metabolic activation to become toxic or, conversely, to be detoxified and excreted. Some metals are nutritionally essential elements at low levels, but are toxic at higher levels (e.g. manganese [Mn]), while others have no known biological functions [e.g., lead (Pb) and arsenic (As)]; because metals naturally occur in the environment, many organisms developed specific mechanisms for its uptake and deposition, specially the accumulation of essential metals. All these particularities can impact the use and interpretation of bioaccumulation data and toxicity of metals (Fairbrother et al, 2007).

1.1.3. Sources of Pb, As and Mn

Pb and As are among the leading toxic agents detected in the environment (Järup, 2003). A recent assessment on the global health impacts of contaminants identified As and Pb

among the six most toxic pollutants threatening human health (Csavina et al, 2012); these elements have been extensively studied, with their effects on human health regularly reviewed by international bodies such as the World Health Organization (WHO) (Järup, 2003). Mn is also of great environmental and public health significance due to its broadly use in many areas of the metal industry (ASTDR, 2007b); in addition Mn (along with Pb and As) is included in a list of metals of primary interest for environmental agencies like EPA (Fairbrother et al, 2007).

Pb is naturally present in the earth's crust where it may exist in three oxidation states, Pb (0), Pb (II) and Pb (IV). In the environment, Pb primarily occurs as Pb (II)' while the occurrence of Pb (0) is less common and Pb (IV) only forms under extremely oxidizing conditions. Organic Pb compounds can also be found as organolead (II) but organolead chemistry is dominated by the tetravalent (IV) oxidation state. Due to its low melting point, easy molding and excellent corrosion resistance Pb is extensively used by men (ASTDR, 2007c). Pb usage may have begun prior to 2000 B.C., when abundant supplies were obtained from ores as a by-product of smelting silver (Casarett and Doull's, 2007). Excessive releases of toxic trace metals into the environment associated with health implications only became apparent in the 1960s and merely when anthropogenic Pb contamination of the urban environment was denoted. In that decade Patterson (1965) wrote that ''the industrial use of Pb is so massive today that the amount of Pb mined and introduced into our relatively small urban environments each year is more than 100 times greater than the amount of natural Pb leached each year from soils by streams and added to the oceans over the entire earth '' (Wong et al, 2006). During the last century the majority of Pb emissions to ambient air resulted from burning gas, coal, oil and waste with over 50% of these emissions originated from petrol. It is estimated that body burdens of Pb in humans in the modern era are 1000-fold higher than during the preindustrial era (Finkelstein et al, 1998). Fortunately over the last few decades, Pb emissions in developed countries have markedly decreased and happened mainly due to the abolishment of leaded gasoline, with a subsequent decrease of blood Pb levels in the general population (Järup, 2003). However, since Pb is a persistent element (Patrick, 2006) past emissions are still a matter of concern. It is estimated that the combustion of

leaded gasoline has accounted for 90% of the Pb that until today is still deposited in the atmosphere. Moreover a number of developing countries continue to use leaded gasoline, still contributing to Pb elevated levels in the atmosphere and other human activities, such as metal smelting, also contribute to significantly release Pb in atmosphere, (ASTDR, 2007b). Airborne Pb can be deposited on soil and water, reaching humans via the food chain. Nowadays, drinking water is a great source of Pb exposure, estimated to be responsible for approximately 20% of the total daily exposure experienced by the majority of the U.S. population (Patrick, 2006).

The metalloid As is particularly difficult to characterize as a single element because it has a complex chemistry and there are many different As compounds, with different oxidation states, each one inducing different toxicities. This element may naturally occur in rocks, soil, water and air (Järup, 2003; Rodríguez et al, 2003) being present as trivalent or pentavalent forms; concerning toxicity, the trivalent form is the most toxic. The most common inorganic trivalent As compounds are As trioxide, sodium arsenite, and As trichloride. Pentavalent inorganic compounds may occur as As pentoxide, As acid, and as arsenates, such as Pb arsenate and Ca arsenate. Organic As compounds may also be trivalent or pentavalent and occur in methylated forms resulting from biomethylation in living organisms, such as mammals, and also in others which are present in soil, fresh water and seawater. When compared with inorganic As, the methylated metabolites are less reactive with the tissue constituents, less acutely toxic, less cytotoxic, and more readily excreted in urine (Casarett and Doull's, 2007; Jomova and Valko, 2011). Due to its wide distribution in Nature arsenical compounds have been known since primordial times and were used in ancient Greece and Rome as cosmetics, therapeutic agents but also as poisons. During the Middle Age and Renaissance, As continued to be used as a poison namely in France and Italy, until the discovery by Marsh in 1836 of a sensitive method to detect As in the tissues of the victims (Rodríguez et al, 2003). Nowadays, the public health concerns pertaining to As are different, and regards to chronic exposures (Hughes et al, 2011; Moinuddin, 2004). Indeed due to the presence of As in the environment, attributable to natural (e.g. geothermal discharges) and anthropogenic sources (e.g. fungicides, industrial products and wastes), exposure of millions of persons to As-

contaminated water is a major concern in many Asiatic countries, where the largest population at risk by groundwater As contamination is in Bangladesh. Several other countries deal with similar harms, namely India, Cambodia, Myanmar, Nepal and Vietnam (ASTDR, 2007a; Kakkar and Jaffery, 2005; Jarup, 2003). Smelting of non-ferrous metals and the production of energy from fossil fuel are the two major industrial processes that lead to As contamination of air, water and soil. Smelting activities are the largest anthropogenic source of atmospheric pollution (Jarup, 2003), being estimated that 40% of the total atmospheric emissions of As arise from these operations (Csavina et al, 2012). Other sources of contamination are industrial products and wastes such as, agricultural pesticides (insecticides and fungicides), wood preservatives and antifouling paints (Jarup, 2003; Kakkar and Jaffery, 2005; Martinez-Finley et al, 2012).

Mn is the 12th most abundant element in Nature and represents approximately 0.1% of the earth's crust. In its natural form, Mn usually exists as oxides, carbonates, and silicates, with Mn dioxide the most commonly found (Martinez-Finley et al, 2012). This transitional metal can exist in 11 different oxidation states, from III to VII, being the most common valence in biological systems is II (Casarett and Doull's, 2007). Mn is an essential element and despite only few cases of Mn deficiency have been reported in humans, its deficiency can lead to serious health disorders (Santamaria, 2008). However, when in excess Mn may also cause a wide range of deleterious effects (ASTDR, 2007c) being the diet, drinking contaminated water and inhalation the main sources to the general population (Wright et al, 2006). In recent years this metal has received increasing attention, since there have been indications that it could become a successor to Pb in urban settings in terms of pervasiveness and long-term adverse health effects (Wong et al, 2006). Indeed, organic Mn is becoming highly present in the environment through the substitution in several countries of leaded-gasoline for the antiknock gasoline additive methylcyclopentadienyl manganese tricarbonyl (MMT). The European Union and Japan have thus far opted against MMT but the compound was approved for use in Argentina, Australia, Bulgaria, Canada, Russia, and conditionally in New Zealand. Also in the U.S., it was decided that the use of MMT is compatible with catalytic converters and has granted a waiver for its use in unleaded gasoline (Gulson et al, 2006; Menkes and Fawcett, 1997). MMT usage has

already been associated with increased Mn atmospheric concentrations (Gulson et al, 2006) and elevated levels of Mn in children's blood have been detected in regions where the additive is allowed (Batterman et al, 2011; Gulson et al 2006). Nowadays, environmental exposure to Mn, through MMT, is an emerging issue still with little information (Batterman et al, 2011).

1.2. Occupational exposure to chemical mixtures

Occupational illnesses are adverse health conditions, occurring with different types of severity and related to chemical or physical factors during work. An occupational illness may develop over time following repeated exposure to a hazard, such as a chemical, and the consequent clinical manifestations may vary with the dose and time of exposure. In this perspective, Occupational Toxicology applies the principles and methodology of toxicology toward chemical hazards encountered at work. Sometimes, health problems become clinically apparent long after the exposure, even after employment has ended, turning difficult the treatments and the establishment of the causal link between the past exposures and the chronic diseases and/or diseases with long latencies, such as are the neurotoxic outcomes. Therefore anticipation is crucial in these contexts, being the main objective of Occupational Toxicology to prevent adverse health effects in workers that arise from their work environment (Casarett and Doull's, 2007).

Millions of workers are employed in manufacturing, mining, construction and other industries, where significant amounts of airborne metals and metal compounds are generated and may cause occupational illness (Ashley, 2009; Félix et al, 2013). Many workers are exposed to high levels of Pb during activities like the manufacture of batteries, sheet lead, bronze plumbing, ceramic glazes, caulking, radiation shields, circuit boards and military equipment (Patrick, 2006) and low or moderate exposure may also take place in the glass industry (Järup, 2003). Occupational exposures to As occurs as well

in several industries, particularly in nonferrous smelting, electronics, wood preservatives, glass manufacturing and application of arsenical pesticides (Kakkar and Jaffery, 2005). The most prominent occupations involving exposure to Mn are mining, namely during ore-crushing, ferromanganese production, smelting and welding (Weiss, 2005). Mn is also found in high concentrations in various other industrial settings, as it is used in the manufacturing of dry-cell batteries, production of Mn-containing organic pesticides, fireworks, ceramics, glass, leather, textiles, paint and cosmetics (Martinez-Finley et al, 2012; Nelson et al, 2012). Descriptions exist in these settings where excessive Mn exposure is associated with several negative health outcomes (Wright et al, 2006).

1.2.1. Occupational exposure in mines

Mining is one of the most hazardous activities for workers (Coelho, 2013) being this occupation long recognized as arduous and liable to injury and disease; the reason is that miners are subject to physical, chemical, biological, ergonomic and psychosocial occupational health hazards, during the mining and also during the associated metallurgical processes (Donoghue, 2004). In the perspective of mining tasks, mineral production can be divided into two general phases: the extraction phase, which consists on removing material from the earth, and the processing phase which separates the mineral component from the ore. Commonly but not quite correctly, the word “mining” is used to describe the phase of extraction, whereas processing describes the phase of preparing the product for human use, but usually the people involved in both processes are called miners or mineworkers (Dahtrak and Nandi, 2009). During both tasks workers are exposed to large amounts of mine dust with various potentially toxicants for eight hours/day and six days/week over a lifetime (Donoghue, 2004). Low-dose chronic exposure to toxic substances results in the accumulation of toxicants in the body (Dahtrak and Nandi, 2009); since some degree of minerals processing is usually undertaken at mine sites, it happens during the processing of ore but also during the associated metallurgical processing, which may involve great risks because air concentrations of pollutants exceed

even those experienced during the mining of the ore. Furthermore, heat and humidity are in deep underground mines, where the virgin rock and air temperatures increase with depth, due to the geothermal gradient and auto-compression of the air column (Donoghue, 2004). Thus, in mining environment, where dermal and inhalational exposures predominate (Dahtrak and Nandi, 2009), inhalation is the primary route of metals exposure (Fairbrother et al, 2007), and the intensity of exposure is often higher due to increased respiration rates (Dahtrak and Nandi, 2009). Bearing in mind that mixtures of neurotoxic metals are present in these settings, this is a quite relevant matter because in the olfactory epithelium the dendrites of the primary olfactory neurons are in contact with the nasal lumen, and these neurons are connected to the olfactory bulbs in the brain. Consequently, materials which come into contact with the olfactory epithelium can be transported to the olfactory bulbs and even further into other areas of the brain (Tjälve and Henriksson, 1999). The most commonly studied occupational health problems of miners all over the world are silicosis, coal workers' pneumoconiosis, asbestosis, etc., but there is a need to study other effects of toxic materials in the mining environment. Whereas acute toxicity is rare in the mining industry, low-dose chronic exposure may result in insidious poisoning that may manifest clinically at a very late stage, and cases of miners with elevated blood levels/body burden of toxicants with vague/no symptoms are not rare. In these cases traditional early warning symptoms of toxicity are often nonclassical because of subclinical toxicity and the possibility of multi-toxicant effects (Dahtrak and Nandi, 2009). It is hence considered vital to monitor mine workers for low toxic levels of mixtures so that subclinical poisoning may be identified at an early stage (Dahtrak and Nandi, 2009; Kakkar and Jaffery, 2009).

1.2.2. Mining activity as a relevant source of exposure to Pb, As and Mn

Mining is one of the oldest activities in human civilization and still a vital economic sector for many countries (Coelho et al, 2013). However, mining operations pose a great potential risk to the environment and to human health (Ericson et al., 2008) since

constitute a significant contributor to pollution of the air, soil and water with a complex mixture of metals (Moreno et al, 2010). In a recent comprehensive assessment of the worst environmental pollution problems, activities associated with mining operations were identified as fourth of the world's top ten pollution problems (Erikson et al, 2004). Mining is a process that begins with the search of mineral deposits and continues with its exploration through ore extraction and processing, which consist of excavating, crushing, grinding, separation, smelting, refining and management of tailings. It also includes the closure and the remediation of worked-out sites (Ericson et al., 2008; Csavina et al, 2012). The environmental and the health impact occur at all of these stages (Ericson et al., 2008) because the vast majority of mining operations produce coarse dusts at high temperatures, potentially laden with the metals and metalloids that are present in the ore. The release of coarse dusts does not occur only in the mine area, but also happens during the transportation of ore with haul trucks and trains (Csavina et al, 2012). Pb, As and Mn are among the major toxicants present in mining environments (Basu et al, 2011; Choudhury and Mudipalli, 2008; Dahtrak and Nandi, 2009; Yim, 2006) with workers and populations living near mining sites exposed to soil and dust containing higher levels of this toxic mixture (Rodríguez et al, 1998). Accordingly, Pb and As contamination have been reported in smelter and mining areas located in different countries, such as Poland, Russia, United States, Mexico, Bolivia, Chile, Peru, Brazil, Philippines, Zimbabwe and Tanzania (Calderon et al, 2003). Water contamination with these elements was reported in Zambia (Ndilila et al, 2013) as well as in soil, air and household dust in Mexico (Calderon et al, 2003). Also the analysis of the components of a mining waste in Villa La Paz (México), revealed the presence of the three elements, Pb, As and Mn (Espinosa-Reyes et al, 2014). In Europe about 2.75 billion tons of mining wastes have been produced between 1998 and 2001. Mining and quarrying waste account for 15% of the total waste in Western Europe and for 31% in the former Eastern European countries (Grangeia et al, 2011).

1.2.3. Mining activity in Portugal

In Portugal, mining and quarrying activities generated 17 Mt in 2001 representing 58% of the total industrial waste (Grangeia et al, 2011) and nowadays, mining still plays an important role in both local and Portuguese economies. However, in some locations the activity is still performed in an uncontrolled way, giving rise to serious environmental contamination. More than a few environmental studies performed in mines and mining surrounding areas in Portugal report anomalous concentrations of metals and metalloids in stream sediments, superficial and groundwater from local courses, road dust, soils and plants for human consumption from nearby villages (Coelho et al, 2013). Soils from the abandoned Pb mine in Braçal had concentrations of Pb higher than the values allowed for agricultural practice (Anjos et al, 2012) and the analysis of heavy metals in wild flora soils suggested that metal contamination, especially with Pb, is a matter of great concern in the Barbadalhos area (Pratas et al, 2013). Also in the abandoned Ribeira mine in the northeast of Portugal, the highest degree of pollution was for As (Ferreira da Silva et al, 2009a) and another area near a closed mine in northern Portugal as well as in abandoned uranium mines in Cunha Baixa were highly contaminated with Mn (Carvalho et al, 2013; Neves et al, 2012). Contaminations with binary mixtures of these elements are also reported in several regions of Portugal. In Coval da Mó mine a huge contamination with Pb (with mean values exceeding 376 times background values) was found in water and Mn was also present in high concentrations (Ferreira da Silva et al, 2009b). The levels of Pb and As in surface water near Panasqueira mine exceeded the reference limits for water quality proposed by the Portuguese Law (Grangeia et al, 2011) and surface waters near the Lousal mine were also contaminated with these elements (Silva, 2009). High concentrations of Pb and As were recorded in the soil near the mine of S.Domingos (Pereira et al, 2004) and water contamination with As and Mn was reported in northern Portugal mining areas, near old Sb-Au mines (Carvalho et al, 2013). The co-occurrence of the three metals, Pb, As and Mn, in higher levels was described in stream sediments that received direct drainage from an abandoned uranium mine in Pinhal do Souto, in Central Portugal (Neiva et al, 2013). Some authors mention that Portuguese mining

activities may adversely affect the health of communities living near mine sites and also the health of miners (Coelho et al, 2013), who are exposed to higher levels of these toxic elements. These informations justify undoubtedly the need to assess the health consequences for workers from Portuguese mines. In Portugal higher levels of Pb, As and Mn were already determined in biological matrices of workers and populations living near the Panasqueira mine (Coelho et al, 2013). In Southeast Alentejo higher concentrations of As and Mn were recorded in peripheral samples of individuals living near a mine and also in subjects that frequently consumed milk and cheese obtained from cattle breeding in the area (Pereira et al, 2004).

1.3. Toxicity of metals

Metals can bioaccumulate being stored in both soft and hard tissues of living organisms (Martinez- Finley et al, 2012) and thus, even the exposure to low levels can lead to long term toxic effects (Dahtrak and Nandi, 2009). Metals disrupt metabolic processes by altering a number of homeostatic processes, which may include antioxidant balance, binding to free sulfhydryl groups (-SH) and compete for binding sites on enzymes, receptors and transport proteins (Martinez-Finley et al, 2012). Pb is considered a dangerous toxicant (Sansar et al, 2011) that may cause neurotoxicity, hypertension, anemia, renal impairment and interfere with sperm production (ASTDR, 2007b; Reckziegel et al, 2011; Patrick, 2006). Pb compounds are reasonably anticipated to be human carcinogens based on limited evidence from studies in humans and sufficient evidence from animal studies (ASTDR, 2007b). The metalloid As can induce cancer, genotoxicity and affect the hematopoietic system, liver, kidneys, skin and brain (Halatek et al, 2009). Exposure to As through drinking water may cause stomach and intestine irritation as well as decreased production of red and white blood cells. The exposure to As has also been reported to increase the risk of cancer in skin liver, bladder and lungs. Long-term Mn exposure is usually associated with central and peripheral nervous system

disorders. Reproductive outcomes might be affected, with decreased libido, impotence and sexual dysfunction. There is no evidence that Mn causes cancer in humans as well as no firm conclusions can be drawn from studies with animals suggesting carcinogenic effects (ASTDR, 2007c).

1.3.1. Neurotoxicity

Neurotoxicity may be defined as any adverse effect, permanent or reversible, on the structure or function of the central and/or peripheral nervous system induced by a biological, chemical or physical agent that diminishes the ability of an organism to survive, reproduce or adapt to its environment (Costa, 1996; Costa and Manzo, 1995). The nervous system can in truth compensate for the toxic effects caused by low doses of neurotoxicants, but a prolonged or lifetime exposure even to its very low levels, can lead to delayed neurodegenerative effects (Lucchini and Zimmerman, 2009), with a progressive loss of neural tissues (Rachakonda et al, 2004). That way neurotoxic effects can be seen as abiding legacies of processes whose roots lie buried in events or conditions occurring even decades earlier and frequently emerging after long latencies. The properties that clinically identify them may bear no more than a superficial resemblance to those manifestations marking their prior stages, explaining why the earliest stages of such diseases may be confused with some other sources, like aging (Weiss, 2006). Neurotoxicity is a sensitive endpoint due to the unique and critical role of the nervous system in the control of body function, including the endocrine and the immune systems. Besides the limited ability of neurons to regenerate after injury, explains neurodegenerative disease related loss of function, as neurons die (Emerit et al, 2004; Mutii, 1999). These disorders may not be healed (Rachakonda et al, 2004) and hence the consequences of neurotoxic effects do not sweep through populations like influenza and then retreat. Rather, they are gradually progressive and the ability of their victims to effectively and efficiently function will be impaired at stages of the disease far earlier than its eventual detection (Weiss, 2006). Thus, an apprehension exists that in a near future

low-dose long-term exposure to metals may give rise to a society where lifelong loss of intelligence and motor capacities, permanent psychological disturbances and disruption of behavior shall be frequently prevalent, giving rise to an unhealthy stressful generation (Kakkar and Jaffery, 2005). These effects can produce reduction of economic productivity and the resulting economic impact may be even greater than the costs of metal pollution control itself (Landrigan et al, 2006).

Chronic exposure to low levels of metals significantly contribute to neurological diseases in multiple populations around the world (Christensen, 1995; Wright and Baccarelli, 2007). Many literature data demonstrate increased levels of metals in critical brain areas of neurodegenerative disease patients (Migliore and Coppedè, 2009). The brain may sometimes compensate for the effects of an individual chemical itself acting on a particular target system; inversely, when multiple targets or functional sites within one system are affected by different mechanisms (such as in multi-metal exposures) homeostatic capabilities may be impaired, thereby leading to cumulative damage (Lucchini and Zimmerman, 2009). The actual public health concern on the potential for exacerbated cognitive and behavioral deficits resulting from children's exposure to multiple toxic metals is an example, with investigations on the effects on cognition of at least two metals together suggesting that combinations of metals may result in increased toxicity at this level (Kordas et al, 2010). Even so, the effect of mixture interactions on neurotoxicity remains largely unknown (Tiffany-Castiglioni et al, 2006).

1.3.1.1. Neurotoxic effects induced by Pb, As and Mn

There is conclusive evidence from experimental and epidemiological investigations that Pb, As and Mn can induce neurotoxicity. The three metals accumulate in the brain (ASTDR, 2007a,b,and c) and the central nervous system (CNS) is a primary target for Pb and Mn (Santos, 2008; Yadav et al, 2011) being neurotoxic effects considered as their critical endpoints (Dukhande et al, 2006; Landrigan et al, 2007; Reckziegel et al, 2011; Reddy et al, 2003; Rodríguez et al, 2010). Pb and Mn induced neurotoxicity is mainly

associated with a CNS dysfunction, whereas As toxicity has been often associated with peripheral nervous system (PNS) alterations. However As CNS effects have been reported in experimental models and humans (Calderon et al, 2003). Additionally, the three neurotoxic elements share common mechanisms of toxicity such as the induction of oxidative stress (OS) (García-Chávez et al, 2006; Patrick, 2006; Zhang et al, 2004) a condition to which the CNS is highly sensitive (Migliore and Coppedè, 2009). Therefore, Pb, As and Mn are relevant elements in the perspective of neurotoxicity induced by mixtures of metals and studies on the interaction of the mixture of these elements are certainly necessary.

Lead

The CNS is a primary target for Pb toxicity (Finkelstein et al, 1998), with the analysis of deceased Pb smelter workers leading to find that Pb accumulation in brain was higher than not occupationally exposed persons (Gerhardson et al, 1995). In cases of exposure to high levels of Pb acute encephalopathy may occur with failure of the blood brain barrier (BBB) function (Lockitch, 1993). This structure is highly vulnerable to the toxic action of Pb and once damaged, Pb itself and other toxicants can easily enter in the brain (Zheng et al, 2003), which in the perspective of exposure to mixtures in occupational contexts is a relevant event. In vivo experiments also showed that the cerebral cortex and the basal ganglia are intensely affected by Pb poisoning. Pb exposure may lead to basal ganglia degeneration, resulting in impairment of neuromuscular coordination and motor control, being actually motor activity in open-field a behavioral parameter experimentally used to assess Pb-induced behavioral toxicity (Moreira et al, 2001). Indeed, performed experiments already leaded to observe smaller number of crossing and rearing movements in rats (Reckziegel et al, 2011) but also sensory function impairment was observed even at low concentrations (Mameli et al, 2001). In addition, because Pb exposure can affect virtually all the neurotransmitters in the brain, such as dopaminergic, cholinergic and glutaminergic systems, most frequently early symptoms of

Pb neurotoxicity include varied signs such as irritability, fatigue, depression, head-ache, decreased attention, memory loss and low-level cognitive impairment (Patrick, 2006).

Despite there is no direct animal test parallel to human IQ tests, a wide variety of assays performed in animals to assess attention, learning and memory suggest that Pb exposure of animals leads to a global deficit, in the same way as it is indicated by decrements in IQ scores in exposed children (ASTDR, 2007b; Finkelstein et al, 1998; Patrick, 2006). Children exposed to Pb may also exhibit symptomatic manifestations of the clinical Attention Deficit Disorder (Finkelstein et al, 1998; Menkes and Fawcett, 1997) along with delinquent behavior, sensory effects (such as hearing and vision) and deficits in neuromotor function (ASTDR, 2007b; Finkelstein et al, 1998). These manifestations were accessed with high Pb body burden conditions, but also with lower blood Pb values such as near or below 10 μg/dL (ASTDR, 2007b; Finkelstein et al, 1998; Patrick, 2006). Pb can also affect the PNS with children having blood Pb levels as low as 0.96 μmol/L exhibiting subclinically impaired peripheral nerve function, shown by slowing the motor nerve conduction (Lockitch, 1993). In adults a most common neurological symptom of Pb exposure is peripheral neuropathy involving extensor muscle groups (Lockitch, 1993; Patrick, 2006) and weakness or paralysis of the wrist, with histopathology analysis showing segmental axonal demyelination and degeneration (Lockitch, 1993). This situation is more common in chronically exposed workers, where blood Pb concentration exceeds 14 μg/dL (a possible threshold). Postural sway abnormalities and essential tremor, particularly for those with genetic susceptibility to Pb exposure, is also documented. It is recognized that past occupational exposure to Pb increases the risk of developing amyotrophic lateral sclerosis and motor neuron disease (ASTDR, 2007b).

Another matter of concern related with chronic exposures to Pb is the fact that the metal can accumulate in bones for 20 years or more (Casarett and Doull's, 2007) and may be released long after the exposure has ceased, due to age-related increases in the rate of bone breakdown (van Wijngaarden et al, 2011). Animal studies showed that mobilization of Pb from bone co-occurs with increased levels in the brain (Finkelstein et al, 1998). That

way, past exposure to Pb continues to be a concern later in life even in the absence of concurrent external exposure sources, with increasing evidences that Pb exposure may contribute to age-related cognitive deficits (ASTDR, 2007b). While chelation is the conventional recommendation for acute Pb toxicity with encephalopathic damage, treatment for chronic low-level exposure is still under investigation and issues surrounding the consequences of low-level environmental exposure are still required (Patrick, 2006).

Arsenic

The effects of As in nervous system have received considerably less attention than other systems (Rodríguez et al, 2003) and thus to date, limited information exists about the effects of As exposure as well as about cellular and molecular mechanisms of the induced neurological effects (García-Chávez et al, 2004). The most consistent behavioral change in rats after As administration—as arsenic trioxide, sodium arsenate or sodium arsenite — is decreased in motor activity (horizontal and vertical parameters) (Yadav et al, 2009), although some neurobehavioral studies also showed that mice exposed to arsenic trioxide can have a biphasic response on motor activity. Indeed, a low dose of arsenic trioxide (3 mg/kg) was able to increase motor activity, while a high dose (10 mg/kg) leaded to it decrease (Rodríguez et al, 2001). Changes within the basal ganglia may be involved in these effects, but today there is not enough data to attribute the observed behavioral changes to a specific action of As on any particular region of the brain. Other CNS functions also seem to be impaired by As, such as learning and memory, with rats exposed to sodium arsenate showing a delay in the acquisition of an operant task (Rodrigues et al, 2003; Flora et al, 2009). In humans As neurotoxic effects are described in children chronically exposed via drinking water (Batterman et al, 2011) and also in exposed workers (Flora, 2011).

Since the first descriptions of neurological injury caused by As in humans were mostly case reports of peripheral neuropathies following its inhalation or ingestion (Rodrigues et

al, 2003), the effects of As in the PNS are better documented than in the CNS (Yadav et al, 2009). Indeed several works described symmetrical peripheral neuropathy, with sensory nerves more sensitive than motor nerves, along with neurons with large axons being more affected than the ones with short axons. Histological examinations showed that axonopathy and demyelination are the main changes in nerves induced by As and a detailed inspection of the distal portion of the nerves showed fragmentation and resorption of myelin. In some nerves there was a reduction, fragmentation or complete degeneration of the axons (Rodrigues et al, 2003). Other cases refer impaired conduction velocity in peripheral motor or sensory nerves (Halatek et al, 2009) and clinical symptoms, such as numbness and paresthesia of the distal extremities (Halatek et al, 2009; Rodrigues et al, 2003; Yadav et al, 2009). Peripheral neuropathy was also present in 15% of residents from a community immediately surrounding a pesticide packaging plant that released As-containing compounds in the form of windblown dust for several years (from 1925 to 1985). These individuals showed significant differences in grip strength, finger tapping, hand–eye coordination and tremor. This study was conducted in 1996 thus showing that neurological deficits in the PNS after chronic exposure to As can be long lasting. In occupational contexts smelter workers showed a significant correlation between decrement of nerve conduction velocity in peripheral nerves and exposure to 50 g of As/m^3 in air in the working area (Rodrigues et al, 2003).

With respect to the CNS, As easily crosses the BBB and although not much information exists about the precise targets of As in brain, basal ganglia has been shown to be quite vulnerable and As can also have marked effects on corpus striatum, hippocampus and cortex (Yadav et al, 2009). Gharibzadeh and Hoseini (2008) suggested that As exposure may be a risk factor for Alzheimer's disease (AD) by inducing apoptosis in cortical neurons (Rodrigues et al, 2003), having been the exposure to this element also associated with Parkinson disease (PD) (de Vizcaya-Ruiza et al, 2009). Children exposed to As and other metals in San Luis Potosí also showed symptoms of CNS alterations, such as impaired learning and memory, sleep disturbances, abnormal performances and altered latency of auditive evoked potentials (Mejía et al, 1995; Yadav, 2009). In patients with occupational exposure to As, encephalopathy in cases of acute exposure was already observed and

description exist about impairments of superior neurological functions such as learning, recent memory and concentration (Mejía et al, 1995; Rodrigues et al, 2003). Exposure to As can also lead to behavioral alterations and namely, a patient who repaired a smelting furnace without respiratory protection exhibited disorientation, severe agitation, paranoid ideation and emotional liability (Rodrigues et al, 2003). Also, Morton and Caron (1989) described the case of two workers occupationally exposed to As fumes that displayed increased irritability; in one worker the symptoms improved 3 months after cessation of the exposure while in the other, four weeks after the exposure the urinary As level was within normal limits. In some cases the severity of the symptoms can be described as related to the duration of the exposure and the symptoms tend to disappear after the end of the exposure (Rodrigues et al, 2003), while in other cases the effects are irreversible (Flora, 2011).

Manganese

Mn presents a special conundrum for risk assessment because is a potent neurotoxicant but it is also an essential nutrient (Weiss, 2005). Mn is required for many essential enzymatic reactions (Wright and Baccarelli, 2006) having a role in a variety of metabolic functions including those involved in skeletal development, energy metabolism, activation of certain enzymes, immunological system, reproduction hormone and also in nervous system function. In the brain, Mn is a cofactor of several enzymes, including the antioxidant enzyme superoxide dismutase (SOD) and also enzymes involved in neurotransmitter synthesis and metabolism (Santamaria, 2008). Thus, a deficiency in Mn can lead to serious adverse effects involving the CNS, such as the impairment of neurological functions, seizures and mental retardation (Wong et al, 2006).

On the other hand Mn can be toxic to several organs, where the critical target organ is precisely the CNS, with induced adverse effects documented at lower levels of exposure comparing with other systems (Health Canada, 2008). In vivo studies reveal that following manganese chloride ($MnCl_2$) administration striatal Mn concentrations could increase by

205%; in another experiment, after exposure to Mn phosphate and Mn phosphate/sulfate mixture, the Mn concentrations in the striatum reached higher levels than the controls (116 and 147%, respectively) (Normandin et al, 2004). Other experiments with mice demonstrated that repeated injections with Mn resulted in its increase in brain and blood, with levels in brain appearing to persist at consistently higher levels for longer periods. These results suggest that the slowly developing neurotoxicity in response to Mn exposure may be due to a persistent retention of this element by the brain (Gianutsos et al, 1985), being Mn pointed as a possible long-term cumulative neurotoxicant (Jiang et al, 2007). The major behavioral effect induced in exposed rodents upon exposure to Mn is the modification of spontaneous motor activity. Some studies describe an increased motor activity that occurs in the initial phase of toxicity, while others report Mn-induced reduction in motor activity with no initial increase (Health Canada, 2008; Normandin et al, 2004). Mn also induces learning impairments in rodents (Health Canada, 2008).

Epidemiological data suggest that high concentrations of Mn in drinking water may be associated with neurological impairment (Weiss, 2005). Nevertheless neurotoxic effects induced by Mn in humans emerge almost exclusively from inhalation exposure, although this exposure route has not been well studied in rats (Health Canada, 2008). Even so, it is achieved that inhaled Mn bypasses the gut and can enter the brain in two ways: 1) through the olfactory pathways providing a direct path into the brain tissue (Weiss, 2005); 2) and through the lungs (Weiss, 2005). In 1) Mn has been found to be taken up and pass transneuronally to other parts of the brain, with these events previously associated to occupational neurotoxicity induced by the inhalation of Mn (Tjälve and Henriksson, 1999. In 2) Mn is not sent directly to the liver, eliminating the first-pass effect and placing Mn available for direct delivery to the brain. Moreover, absorption within the respiratory tract is not thought to be under homeostatic regulation (Health Canada, 2008).

The recognition of the neurotoxic properties of inhaled Mn came since the 19th century and concerns to the context of mines. As described by Couper (1837) four Mn ore crushers developed lower extremity predominant ‘‘muscular weakness’’, festination, postural instability, facial masking, hypophonia and sialorrhea. The syndrome was more clearly delineated by Rodier (1955) when he described a group of Moroccan Mn miners with a neurologic illness characterized by parkinsonism, gait disorder, dystonia, psychosis and emotional labiality (Nelson et al, 2012). Nowadays this syndrome is broadly documented and is known as “manganism”, a clinical and severely debilitating neurological disease that most frequently occurs in workers exposed through inhalation to very high levels of this metal (generally over 1 mg/m^3), but that may also be induced by prolonged and subclinical exposure (Wong, 2006). Manganism is indeed identified in miners (Ramteke et al, 2009; Weiss, 2005) namely in workers involved in activities such as ore processing and welding (Antonini et al, 2006; Bowler et al, 2006; Weiss, 2005). This progressive syndrome typically begins with relatively mild nonspecific symptoms, which can gradually evolve to a severely debilitating disease (Ostiguy et al, 2005). Two or three clinical phases distinguish the development of chronic manganism. During the prodromal period, the disorders start gradually with symptoms that include asthenia, anorexia, apathy, headaches, hypersomnia, spasms, legs, arthralgia and irritability. This is followed by the intermediate phase in which signs such as speech disorders, clumsiness in movements and masked facial appearance show up. Finally, the late (or established) phase is characterized by muscular rigidity and tremor of the upper limbs (Ellingsen et al, 2008; Normandin et al, 2004), among several other effects described in diverse occupational studies such as memory loss, impulsive-compulsive behaviors, abnormal laughter, psychotic experiences, impaired coordination and bradykinesia (Weiss, 2005; Wong et al, 2006; Wright and Baccarelli, 2006). There are characteristics of manganism that resembles the neurodegenerative movement disorder PD in symptomatology and cellular mechanisms. This includes motor and postural signs consistent with those of PD as well as Mn-induced oxidative stress and also a selective toxicity towards dopaminergic neurons, which are involved in the regulation and control of movement (Normandin et al, 2004). This overlap has led to debates about whether Mn neurotoxicity is truly a form of PD (Wasserman et al, 2011), while some authors defend that enough differences are

apparent to support the distinction (Weiss, 2005). Neurodegeneration in PD occurs primarily in the substantia nigra pars compacta, while in manganism the main evidence of degeneration is seen in the globus pallidus, with the nigrostriatal pathway usually preserved and exhibiting less severe damage in nearby structures including the striatum (putamen and caudate nucleus) and in the substantia nigra pars reticulate (Ellingsen et al, 2008; Weiss, 2005). Additionally, despite in both PD and manganism there is a generalized bradykinesia and widespread rigidity, manganism differs from PD by a notably less-frequent resting tremor, more frequent dystonia, a particular propensity to fall backwards, and a failure to achieve a prolonged therapeutic response to levodopa (Normandin et al, 2004). In any case the subclinical deficits observed in Mn-exposed subjects are consistent with damage of the basal ganglia in the brain (Health Canada, 2008) where its accumulation has been seen in welders (Ellingsen et al, 2008). Risk factors for manganism include liver diseases, that increase the body load of Mn and also alcoholism, whose effects add to the neurotoxic action of Mn (Ostiguy et al, 2005), being important to contemplate that this risk factor is relevant in mining environment ever since high prevalence of alcohol and drugs abuse may occur in these occupations (VWA, 2014). Manganism is considered an irreversible condition, although some evidence exists that in the early stages of intoxication the symptoms may be reversible (Bowler et al, 2006). It is also known that Mn neurotoxicity may manifest even years after the exposure has ceased, being neurological alterations reported for ex-miners in South Africa (Myers et al, 2003a). Nonetheless even considering the available information about manganism and Mn induced neurotoxicity, there is much to be learned and evaluated concerning prolonged exposure to low levels of this metal (Landrigan et al, 2007).

Binary mixtures

Practically no reports are found respecting specifically to the neurotoxic effects induced by the mixture of Pb, As and Mn, except a few experimental works on their binary mixtures demonstrating interactions between the metals. The co-administration of Pb and Mn to rats leaded to observe that the gestational exposure to both elements reduced

brain's weight in a greater extent than either metal alone (Kim et al, 2009a). Pb levels also increased in several brain regions like the cerebellum, cerebral cortex, corpus striatum, hippocampus and midbrain, as compared with the administration of Pb alone (Shukla and Chandra 1987). ln addition Pb increased the concentration of Mn in corpus striatum and midbrain as compared with Mn alone at the same dose (ASTDR 2007c; Shukla and Chandra 1987). Even very low doses of Pb plus Mn raised the striatal Mn and Pb concentrations (Shukla and Chandra, 1987). Behavioral changes were also denoted such as a significant hypoactivity an aggravation of the aggressive behavior induced by Pb (Chandra et al, 1981). Tests of learning ability, that measured conditioned avoidance responses, showed that the mixture of Pb and Mn impaired learning in a greater extent than the single administration of Pb. These results suggest the possibility of serious brain dysfunctions in humans after co-exposure to even subclinical levels of the two metals through a polluted environment (Shukla and Chandra, 1987). Accordingly EPA recommended further assessing of the potential hazard to public health of the joint toxic action of the Mn/Pb mixture, with relevance for the estimation of endpoints for neurological effects (ASTDR, 2004). In humans a positive correlation between Mn and Pb in blood was detected while the co-exposure to environmental Pb and Mn revealed to affect the intelligence of school-aged children (Kim et al, 2009b). A few studies were performed with other binary mixtures, such as the co-administration of Pb and As in rats resulting in increased Pb brain levels, along with decreased As concentrations (Mejía et al, 1997). Neurotransmitter changes were also found over control values, alterations that were not elicited by either metal alone (Carrizales et al, 2006). The mixture of As and Pb showed to induce neuropsychological effects in children living in Morales (Mexico) despite in this study no conclusive results were obtained regarding to interactions between these elements (Carrizales et al, 2006). Some work exists as well with the mixture of As and Mn, where Rodriguez et al (1998) observed that As and Mn had a greater accumulation in rats' brains comparing to animals with single metal exposures. In addition, higher hair As and Mn levels in humans were associated with significantly lower scores on IQs test and also on verbal learning and memory tests. In some cases, a significant Mn and As interaction was determined (Wright et al, 2006). With respect to the mixture of the three elements one study reported that when combined, Pb, As and

Mn were associated with changes in neurotransmitters. The authors concluded that the findings were complex, but the data clearly supported the concept that co-exposure to multiple metals could cause neurotoxic effects not seen with exposure to a single metal at the same dose (Wright and Baccarelli, 2009).

1.4. Mechanisms of neurotoxicity induced by Pb, As and Mn

1.4.1. Oxidative stress

Oxidative stress (OS) is an important convergent point on the mechanisms of metal toxicity (Whittaker et al, 2010) representing a pathway that leads to the destruction of cells, including neurons and vascular cells in the CNS (Chong et al, 2005). This condition seems to be a leading mechanism of Pb (Adonaylo and Oteiza, 1999), As (Jomova and Valko, 2011; Rodríguez et al, 2003) and Mn (Erikson et al, 2004) neurotoxicity and either as a cause or as consequence, and directly or indirectly, OS is related with the biomarkers (BMs) of effect for these metals that will be focused along this work. By these reasons, a particular emphasis will be given to this deleterious condition.

Oxidative stress can be defined as an imbalance between biochemical processes leading to the production of reactive oxygen species (ROS) and those responsible for its removal (Sayre et al, 2008; Wang and Michaelis, 2010; Whittaker et al, 2003). In healthy conditions a wide variety of ROS are produced in cells during the course of the normal metabolism (Migliore and Coppedè, 2009). ROS formation occurs mostly as a byproduct of oxidative phosphorylation (Perry et al, 2002) since under physiological O_2 levels 1–2% of the O_2 consumed is converted to ROS (Emerit et al, 2004; Migliore and Coppedè, 2009). The most common cellular free radicals are superoxide radical ($O_2^{-}\bullet$) and the hydroxyl radical

(•OH). Other molecules, such as hydrogen peroxide (H_2O_2) are not free radicals, but can lead to their generation through various chemical reactions (Gilgun-Sherki et al, 2001). Despite not much referred, ROS have several important functions in cells such as the modulation of critical cellular functions, notably for neurons, astrocytes and microglia, such as mitogene-activated protein (MAP) kinase cascade activation, ion transport, Ca mobilization and apoptosis program activation (Emerit et al, 2004). When excessive ROS accumulation occurs, cells possess an intricate network of defense mechanisms to neutralize them, including antioxidant compounds such as glutathione (GSH), arginine, taurine, creatine, Se, Zn, vitamin E, vitamin C, vitamin A and antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx). Therefore, under physiological conditions, cells are able to cope with the flux of ROS (Lawrence et al, 2005; Maes et al, 2011; Migliore and Coppedè, 2009) and its presence is inclusively a benefit. Oxidative stress rises when an excessive amount of these molecules accumulate and/or when cellular antioxidant defenses are insufficient to keep the levels of ROS below a toxic threshold (Migliore and Coppedè, 2009; Wang and Michaelis, 2010). Since these reactive species contain one or more unpaired electrons they are more reactive and essentially capture electrons from other molecules present in the living tissues (Gilgun-Sherki et al, 2001). Not rarely these reactions result in the creation of a new radical (Qureshi et al, 2004) that can be just as damaging as the initial ROS (Migliore and Coppedè, 2009; Sayre et al, 2008). By this mean, reactive species are detrimental to cells (Emerit et al, 2004) damaging lipids, sugars, proteins, polynucleotides and inactivating enzymes and other proteins or inducing DNA strand breaks (Whittaker et al, 2011). The net result is cell damage and decreased viability of the cell system, leading to critical failure of biological functions and ultimately cell imbalance and death (Perry et al, 2002).

Like other tissues, the brain is exposed throughout life to OS and certain nervous system diseases are thought to involve oxidative damage, either as a primary cause and/or as a consequence of disease progression (Gilgun-Sherki et al, 2001). The CNS is particularly vulnerable to oxidative insult, on account of the high rate of O_2 utilization (Migliore and Coppedè, 2009) which is actually about one-fifth of the oxygen consumed by the body

(Emerit et al, 2004; Lawrence et al, 2005; Migliore and Coppedè, 2009); In the brain there are also relatively poor concentrations of antioxidants and related enzymes like GPx and CAT, along with a high content of polyunsaturated lipids, biomolecules highly susceptible to oxidation (Migliore and Coppedè, 2009); Regionally high concentrations of redox-active transition metals exist, such as Fe, which is capable of the catalytic generation of ROS (Chong et al, 2005); the metabolism of neurotransmitters also produces ROS (Migliore and Coppedè, 2009). For all these reasons it is not surprising that OS is a common discussion point for neurodegeneration (Chong et al, 2005; Gilgun-Sherki et al, 2001; Sayre et al, 2008).

The exposure to environmental chemicals can induce alterations in the cellular redox balance, being exposure to metals an important contribution for the development of oxidative events. Both oxidant-mediated damage and/or depletion of antioxidant molecules has been widely reported as the main mechanisms of metal-induced cytotoxicity (Campbell et al, 2001; Franco et al, 2009; Migliore and Coppedè, 2009), where abnormal tissue accumulation of redox-active transition metals (such as Mn) and also redox inert-metals (such as Pb and As) (Jomova and Valko, 2011; Sayre et al, 2008) induce perturbations of metal homeostasis, resulting an array of cellular disturbances (Campbell et al, 2001; Jomova and Valko, 2011). Pb-induced OS has been identified as the primary contributory agent in the pathogenesis of Pb poisoning (Flora et al 2005; Gurer and Ercal, 2000; Reckziegel et al, 2011), with evidences of oxidative damage in several organs including the brain (Patrick, 2006). Free radical Pb-induced damage is accomplished by two independent although related mechanisms. The first involves the direct formation of ROS including singlet oxygen ($1O_2$), H_2O_2 and also the formation of peroxynitrite ($ONOO^-$). The second one relies on Pb capacity to bind to enzymes with functional -SH groups, directly depleting antioxidant molecules containing these groups (Franco et al, 2009). Pb can deplete SOD, CAT and GPx (Franco et al, 2009; Jomova and Valko, 2011; Patrick, 2006; Reckziegel et al, 2011) leading to decreased reduced glutathione: oxidized glutathione (GSH: GSSG) ratios that will render cells more susceptible to oxidative damage (Gurer and Ercal, 2000). Depressed levels of GR and GPx were found in Pb occupationally-exposed workers (Casarett and Doull's, 2007). Moreover,

Pb conjugates directly with GSH (Franco et al, 2009; Jomova and Valko, 2011), which represents more than 90% of the non-tissue sulphur pool of human body. Reduced GSH levels were already observed in Pb-treated animals as well as in exposed workers (Gurer and Ercal, 2000). Pb is also known to have toxic effects on membrane structures enhancing lipid peroxidation (Ahamed and Siddiqui, 2007).

Oxidative stress is currently the most widely accepted mechanism of As toxicity and is possibly the leading path for their induced neurological defects (Flora, 2011). It is assumed that As can directly attack the mitochondria leading to the generation of ROS, namely via the uncoupling of the oxidative phosphorylation by As (V) (Franco et al, 2009). Concomitantly As species can either induce ROS, which target the mitochondria (Flora, 2001; Franco et al, 2009) through several proposed paths: the generation of intermediary arsine species may produce significant amounts of free radicals, where namely molecular oxygen can react with dimethylarsine to form dimethylarsinic radical [$(CH_3)2As\bullet$] and $O_2^{-}\bullet$ and further, the addition of another molecule of O_2 results in a dimethylarsinic peroxyl radical [$(CH_3)2AsOO\bullet$]; As species may also displace Fe and Cu from metalothionein (MT) and other metal-containing proteins (Jomova and Valko, 2011). In turn, these redox-active metals play a central role in the generation of ROS by promoting the conversion of H_2O_2 into the highly reactive •OH radical through Fenton reactions; under physiological conditions the oxidation of arsenite [As (III)] to arsenate [As (V)] results in the formation of H_2O_2 (Flora, 2001); also As can mediate the formation of singlet oxygen ($1O_2$) and nitric oxide (NO•) (Flora, 2001; Jomova and Valko, 2011). Accordingly, As-induced •OH generation was reported in the striatum of rats (Flora, 2001), with the presence of this reactive species associated with As-induced disturbances in the CNS (Jomova and Valko, 2011). This metalloid can also intensively affects ROS-metabolizing enzymes including SOD, CAT, thioredoxin reductase as well as GPx, glutathione S-transferase (GST) and GR thus, interfering with GSH. Generally short-term exposure to low As concentrations results in increased activity of these enzymes, whereas chronic exposure usually results in their reduction (Flora, 2011; Franco et al, 2009; Jain et al, 2011; Jomova and Valko, 2011). Additional mechanisms exist for As-induced cellular GSH pools depletion, which are: via GSH-mediated reduction of pentavalent to trivalent arsenicals where GSH acts as an

electron donor; direct interaction of GSH with As; and GSH oxidation through As-induced generation of free radicals (Flora et al, 2005; Franco et al, 2009; Jomova and Valko, 2011). It must be emphasized that concerning the exposure to mixtures the depletion of GSH is a quite sensitive event, since may contribute to raise the toxicity of other metals (Jomova and Valko, 2011).

Mn is a very interesting element concerning its OS mechanism of toxicity. At normal levels Mn plays a role against oxidative damage since is a constituent of mitochondrial Mn-SOD, contributing to keep $O_2^-\bullet$ at nontoxic levels. On the other hand, when in higher concentrations Mn may induce OS (Milatovic et al, 2009) since may contribute precisely to the formation of the same species, the $O_2^-\bullet$. In cells the metal preferentially accumulates in mitochondria, where it disrupts oxidative phosphorylation by interfering with the mitochondrial electron transfer and increasing this way, the generation of ROS (Milatovic et al, 2009; Zhang et al, 2004). More explicitly proteins or quinones that participate in the transfer of electrons such as the complex I can be damaged by Mn and consequently the chain begins to donate electrons directly to O_2 leading to the creation of the $O_2^-\bullet$ (Erikson et al, 2004). Additionally the physiological cycling between Mn(II) and Mn(III) can also involve the induction of OS (Casarett and Doull's, 2007), through the following reactions:

$$Mn\ (II) + O_2 \rightarrow Mn\ (III) + O_2^-\bullet$$

$$Mn\ (II) + H_2O_2 \rightarrow Mn\ (III) + OH^- + \bullet OH$$

In the brain, the pro-oxidant effects of both Mn (II) and Mn (III) have been confirmed in vitro and in vivo studies, with the suggestion that Mn exposure enhances the rate of ROS generation in a dose-dependent manner (Erikson et al, 2004). It is also documented that rats exposed to Mn exhibited a significant decrease of GSH levels in striatum and a significant decreases in GPx activity in the cerebral cortex (Huang et al, 2011).

1.4.2. Interference with the dopaminergic system

Dopamine (DA) is a neurotransmitter involved in a variety of CNS processes, like cognition, mood, attention and learning and most particularly, in motor activity (Clavier et al, 2009). Thus, it is not surprising that alterations in DA transmission can adversely affect a variety of neurological processes leading to various debilitating behavioral disorders. Dopaminergic transmission is a complex process that occurs through several linked processes, including synthesis, release, uptake, storage and catabolism of DA. Tyrosine hydroxylase (TH) converts L-tyrosine to L-DOPA which in turn, is decarboxylated to DA by aromatic L-amino acid decarboxylase. Once synthesized, DA is packaged into synaptic vesicles and in response to a presynaptic action potential is released from the cell. Once released, DA activates a variety of postsynaptic receptors which are coupled to various cell signaling mechanisms. DA transmission is inactivated by reuptake of DA via the DA transporter (DAT) into the presynaptic neuron (Jones and Miller, 2008). During this process a relation exists between DA metabolism and OS since DA is easily metabolized via monoamine oxidase (MAO) or by autoxidation to produce cytotoxic ROS (Chong et al, 2005). In the oxidation of DA by MAO, dihydroxyphenylacetic acid and H_2O_2 are generated and because in DAergic neurons transition metals are abundant, H_2O_2 can react with metals, specially Fe, to form the most cytotoxic radical •OH. When non-enzymatical and spontaneous autoxidation of DA occurs, O_2^-• and reactive quinones are produced. To worsen when DA neurons are damaged, an excess amount of cytosolic DA exists outside the synaptic vesicule and is spontaneously oxidized. An interesting note is that DA quinones are also generated in the enzymatic oxidation of DA by cyclooxigenase (COX) (Miyazaki and Asanuma, 2009) and neuroinflammation (a potential source of ROS by itself) is caused by Pb, As and Mn (Kasten-Jolly et al, 2011; Shavali and Sens, 2008; Streifel, 2011).

Neuronal damages induced by Pb may result in changes of DA transmission (Reckziegel et al, 2011), which is demonstrated by several animal studies (Rodríguez et al, 1998; Roses et al, 1989). While neonatal exposure to low doses of Pb may result in increased DA

levels, a large dose leads to decreased levels, which suggests that the dose has a major impact on the effects of Pb on DA neurotransmission (Jones and Miller, 2008). The mechanisms through which inorganic Pb affects neurochemistry and behavior are still unknown (Struiyriska and Rafafalowska, 1994), but most of the available evidence suggests that Pb may impair regulation of DA synthesis and release, indicating a presynaptic site of action (ASTDR, 2007b, Reckziegel et al, 2011). Post-weaning exposure of rats to Pb resulted in hypersensitivity of D1 and D2 dopamine receptors, which can be interpreted as a compensatory response to decreased synthesis and/or release of DA (ASTDR, 2007b). Oxidative damages to the postsynaptic membranes has also been demonstrated (Reckziegel et al, 2011) and additionally Pb(II)/Ca(II) interactions might play an important role in the neurotoxicity of Pb, since Pb can mimic or displace Ca during neurotransmission processes, interacting at sites involved in the release of neurotransmitters. By blocking the influx of Ca ions into the presynaptic terminals during their depolarization by the nerve action potential, Ca-dependent DA release may be inhibited (Struiyriska and Rafafalowska, 1994).

It is acknowledged that As can affect dopaminergic functions (Rodríguez et al, 2010) decreasing DA levels in exposed rats (ASTDR, 2007a), namely striatal DA (Rodríguez et al, 1998); decreased striatal DA was also exhibited in animals treated with mining waste containing a high concentration of As (Bardullas et al, 2009). As-induced changes in brain DA levels were also found to be linked with enhanced OS, being demonstrated that a mixture of As (III) and DA was synergistic concerning its ability to elicit the death of SH-SY5Y cells, a human neuroblastoma cell line that retains dopaminergic differentiation. Raised DA-quinone leaded the authors to propose that As (III) together with DA could produce highly toxic free radicals (Shavali and Sens, 2007). Additionally, when antioxidant defenses are surpassed (the effect of As on antioxidant systems was previously referred) inorganic As may cause alterations at the transcriptional level in neurotransmission systems or in the affinity of DA receptors. Again the formation of ROS may be involved, where H_2O_2 as a by-product of the reaction of inorganic As with oxygen could play a role as a disruptor of DA receptors gene expression, although further studies are necessary to confirm this hypothesis (Rodríguez et al, 2010).

Mn is a potent dopaminergic neurotoxicant which ultimately leads to impairments in DA-associated behaviors, explaining the specific effects on motor, memory and mood functions, usually associated with Mn overexposure (Finkelstein et al, 2007; Takser et al, 2004). Actually, this metal seems to have a selectivity for dopaminergic neurons (Ellingson et al, 2003) where exerts cytotoxic effects that can lead to its degeneration and death (Jones and Miller, 2008; Prabhakaran et al, 2008). Experiments with mice feeded with Mn-enriched pellets showed striatal DA decrease, while other studies with rodents demonstrated decreased motor activity after Mn paralleled striatal DA loss dose–dependent. More than 70% loss of striatal DA was recently reported after experimental exposure to Mn, through inhalation of an ultranebulized mixture of divalent and trivalent forms (Madison et al, 2012; Peneder et al, 2011; Takser et al, 2004). Oxidative stress seems to be involved in these events, more specifically via Mn-induced oxidation of DA (Erikson, 2004; Jones and Miller, 2008). It is believed that free radicals generated by Mn (II) ions increase the oxidation of this catecholamine, a process that is accompanied by an increase of quinones (Jones and Miller, 2008; Prabhakaran et al, 2008) with resulting OS and neurotoxicity, may causing dopaminergic cell death (Zhang et al, 2004). It is also postulated that DA auto-oxidation following Mn exposure activates intracellular signaling cascades, particularly nitric oxide synthase (NOS) potentiating dopaminergic toxicity (Prabhakaran et al, 2008). DAT may also mediate Mn accumulation in dopaminergic terminals or serve as a target for Mn-induced ROS which in turn, can lead to a disruption of DA metabolism (Jones and Miller, 2008; Ramteke et al, 2009). Some authors suggest that increased DAT levels observed in in vivo experiments upon exposure to Mn may be a compensatory response to Mn-induced DAT inhibition and that the transient increase in DAT levels may reflect an early event in Mn neurotoxicity, predisposing the DA cell to oxidative damage (Jones and Miller, 2008). Mn may also interfere with Ca homeostasis affecting in this way Ca – dependent DA release (Ramteke et al, 2009).

Prolactin (PRL) is a polypeptide neurohormone synthesized and secreted from the lactotroph cells in the anterior pituitary gland. Aside from its action on reproduction and lactation, PRL plays important roles in the regulation of the immune system, osmotic balance and angiogenesis (Ellingson et al, 2003; Meeker et al, 2009; Rattanakul and

Lenbury, 2009). PRL is becoming increasingly used as a measure of neuroendocrine/dopaminergic function in environmental and occupational epidemiology studies (Meeker et al, 2009; Takser et al, 2004). In fact DA, along with other compounds, regulate the release of PRL (Meeker et al, 2009) since DA input from the hypothalamic tuberoinfundibular dopaminergic system (TIDA neurons), released into the hypothalamic hypophyseal portal system and bound to D2-receptors of lactotropes, are responsible for the secretion of PRL in the pars distalis (Clavier et al, 2009; Ellingson et al, 2003; Marreiha dos Santos et al, 2011; Rattanakul and Lenbury, 2009) The activation of D2 receptors result in increased K conductance that provokes hyperpolarization and inactivation of voltage-dependent Ca channels (Montes et al, 2011; Takser et al, 2005). A variety of other modulators of PRL secretion (e.g., serotonin and GABA) act at the hypothalamic level inhibiting or reinforcing the dopaminergic tone and by its turn the hypothalamic dopaminergic neurons (Marreiha dos Santos et al, 2011). Down regulation of the expression of PRL gene is an additional mechanism for the control of PRL release by DA (Montes et al, 2011; Takser et al, 2005). Decreased DA turnover in hypothalamus leads indeed to a corresponding increase in PRL levels in rats (Santos et al, 2008) and this evidence in humans has been corroborated by observations that drugs which interfere with DA release affect circulating PRL levels (Marreilha dos Santos et al, 2011).

Pb ingestion may interfere with PRL secretion in pregnant mice leading to aggressive social behavior and again, increased serum PRL was associated to depression of inhibitory dopaminergic influence on pituitary PRL release. Additional mechanisms have been proposed from studies with pregnant women, where a relationship between serum PRL and blood Pb levels could be due to the PRL effect on bone metabolism which increases bone Ca mobilization during pregnancy. Given Pb's capability to substitute Ca (Casarett and Doull's, 2007), Pb mobilization from maternal skeleton could be associated with elevated serum PRL levels (Takser et al, 2005). Also, Pb exposure in fish *Catla catla* caused a decrease in Ca content of bones, which was ascribed to the increased activity of PRL cells and consequent PRL release to restore decreased blood Ca (Srivastav et al, 2013). Biochemical mechanisms underlying Mn toxicity include free radical generation and DA

oxidation (Smargiassi and Mutti, 1999) where elevated serum PRL suggest DA disturbance (Montes et al, 2011).

1.4.3. Interference with the cholinergic system

The cholinergic system is involved in many functions of the nervous system, ranging from vegetative functions (heart rate, blood pressure), to locomotion, muscular contraction and cognition. Thus, the consequences of disturbing this system are complicated and diverse (Finkelstein et al, 2007; Moser, 1995; Shen, 2004). Acetylcholine (ACh) is a neurotransmitter (Richetti et al, 2011) of this system and acetylcholinesterase (AChE) is an enzyme involved in the metabolism of ACh (Yadav et al, 2011). The enzyme plays a role in the cessation of the impulse transmission at cholinergic synapses in the extracellular space, by rapid hydrolysis of ACh (Dvira et al, 2010) into choline and acetic acid. This reaction is necessary to allow a cholinergic neuron to return to its resting state after activation (Ali et al, 2010). Besides currently available evidence obtained from in vivo and in vitro experiments shows that AChE possess many other functions that do not seem to be solely related to their hydrolytic properties and include neurogenesis and its development, synaptogenesis and its development, neurotrophic activity, cell proliferation, cell differentiation, cell adhesion, cell plasticity, signal transduction, regulation of the BBB and glucose energy metabolisms (Shen, 2004). Several chemicals inhibit AChE causing neurotoxicity (Ali et al, 2010) namely through ACh accumulation in the synaptic cleft with overstimulation of muscarinic and nicotinic ACh receptors (Santos et al, 2011). AChE is anchored to the plasma membrane and there is evidence that lipid peroxidation aroused from OS can alter its activity (Ademuyiwa et al, 2007; Santos et al, 2011; Rosenberg et al, 2010). Also AChE inhibition in rats' brain may lead to oxidative events and eventually disrupt the delicate balance between ACh and DA (Finkelstein et al, 2007) in the cholinergic-dopaminergic system, where ACh is thought to act directly on DAergic terminals. Cholinergically induced release of DA that occurs in the presence of tetrodotoxin supports this supposition (Santos et al, 2012).

AChE is found in nervous tissues but also in the erythrocytes (Ali et al, 2010) and thus, measurements of AChE activity in blood are well accepted BMs of exposure to neurotoxic chemicals (Ali et al, 2010; Solé et al, 2008). Erythrocyte-AChE determinations are used in occupational medicine for periodical screening of agricultural workers exposed to organophosphorous pesticides (Finkelstein et al, 2007). Although much less studied than dopaminergic systems, cholinergic functions are also disturbed by Pb (Ademuyiwa et al, 2007), As (Rodríguez et al, 2003) and Mn (ASTDR, 2007c).

Pb exposure affects the cholinergic system mainly by reducing ACh release, uptake and turnover rates (Amal and Mona, 2009). In vitro and in vivo studies have also shown the inhibitory effects of Pb on AChE activity (Ademuyiwa et al, 2007; Finkelstein et al, 1998; Reddy et al, 2003), with whole brain AChE activity showing marked depletions (Tripathi et al, 1997). Some authors attribute arsenite effects to its interaction with the disulfide group of AChE (Flora, 2011; Rodríguez, 2003). In addition As enhances free radical generation and impairs anti-oxidant defenses leading to increased OS in the brain. These events are accompanied by a decrease in cholinergic receptors in the hippocampus and frontal cortex of exposed rats. The described damages were associated with memory deficits observed in the animals (Yadav et al, 2011). Mn seems to exert various effects in multiple sites within the cholinergic neurons in peripheral, motor nerves and neuromuscular junctions (Finkelstein et al, 2007) and also in the CNS. In the brain Mn perturbs cholinergic mechanisms not only in the basal ganglia but also in cortical brain regions such as the hippocampus, the frontal cortex and the parietal cortex. The blocking of the release of ACh by Mn at presynaptic levels is among the proposed mechanisms (Finkelstein et al, 2007), being also suggested that Mn-induced AChE targeting may trigger or contribute to the development of OS. In vivo experiments already showed decreased brain AChE activity accompanied by higher levels of indicators of peroxidation in membranes (neuro and isoprostanes) upon Mn exposure (Santos et al, 2012).

1.4.4. Interference with the hematopoietic system

Pb, As and Mn can exert several effects in the hematopoietic system (Case et al, 2013; Cory-Slechta, 1990, Pereira et al, 2010), which includes interferences with heme synthesis, may leading to neurotoxicity (Demasi, 1996, Flora et al, 2005; Reckziegel et al, 2011). Heme is an indispensable cofactor in oxygen transport and storage (hemoglobin, myoglobin), mitochondrial electron transport (complexes II–IV), drug and steroid metabolism (cytochromes), signal transduction (nitric oxide synthases, soluble guanylate cyclases), transcription and even regulation of antioxidant-defense enzymes (CAT and GPx) (Krishnamurthy et al, 2007; Ryter and Tyrrell, 2000). The synthesis of heme occurs in every human cell, although about 85% occurs in the bone marrow, where the majority is used for hemoglobin formation (Hift et al, 2011; Kauppinen, 2005; Quintanilla-Vega et al, 1996). In this pathway succinyl-coA and glycine are combined under the enzymatic control of delta-aminolevulinic acid synthase (ALAS) to form delta-aminolevulinic acid (ALA). Subsequently, two molecules of delta-ALA are condensed to form the pyrrole ring structure of porphobilinogen (PBG), catalyzed by ALA dehydratase (ALAD). Collectively ALA and PBG are known as porphyrin precursors (Hift et al, 2011). Four PBG molecules are then joined by hydroxymethylbillane synthase (HMBS) to form hydroxymethylbillane (HMB), a tetrapyrrole ring structure. The majority of HMB is converted to uroporphyrinogen III by uroporphyrin synthase although there is a lesser amount of non-enzymatic conversion of HMB to uroporphyrinogen I and then to coproporphyrinogen I. Uroporphyrinogen III is decarboxylated by uroporphyrinogen decarboxylase to coproporphyrinogen III, which then enters in the mitochondria for the final steps of heme synthesis. Protoporphyrinogen IX is formed after oxidative decarboxylation of coproporphyrinogen III by coproporphyrinogen oxidase and subsequently Fe is added to the molecule by ferrochelatase (FECH) to form heme (Simon and Herkes, 2011) (Fig.1.1).

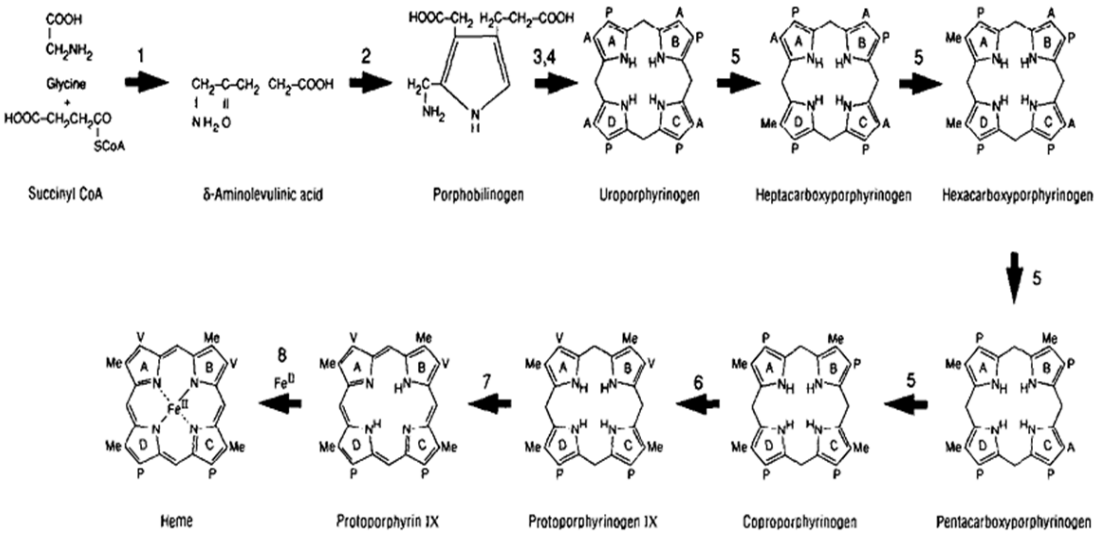


Fig. 1.1: Heme biosynthetic pathway. Steps are catalyzed by (1) d-aminolevulinic acid synthetase (ALAS), (2) ALA dehydratase (ALAD), (3) uroporphyrinogen I synthetase, (4) uroporphyrinogen III cosynthetase, (5) uroporphyrinogen decarboxylase, (6) corporpophyrinogen oxidase, (7) protoporphyrinogen oxidase, and (8) ferrochelatase. Adapted from Woods et al, 1991.

Eight enzymes catalyze these reactions (Hift et al, 2011; Kauppinen, 2005) and most importantly, those enzymes have been shown to be specifically susceptible to impairment by a variety of toxic agents (Bleiberg et al, 1967; Quintanilla-Vega et al, 1995). These agents includes metals such as Pb and As (Ng et al, 2005), due to its strong affinity for their functional -SH groups (Flora, 2011; Woods et al, 2009); concerning Mn little information exists related with the impairment of these enzymes (Maines, 1980). When interferences occur in the heme biosynthetic pathway, there is characteristically an excessive accumulation and excretion of ALA and/or porphyrins (Adhikari et al, 2006; Guolo et al, 1996). Because individual porphyrins differ by the side chain substituents, different metals may induce specific and different changes in porphyrin excretion patterns (Woods et al, 2009). Thus, the urinary porphyrins profile became a key tool for the diagnosis of heme biosynthetic pathway disturbances (Bozek et al, 2005) and may serve as a BM of exposure to toxic metals in humans (Li et al, 2011; Ng et al, 2005). The circumstance that different metals can exert their effects at different points of this metabolic pathway is a curious particularity that justifies the opinion of some authors,

who mention porphyrins as promising BMs of toxicity induced by metal mixtures (Wang and Fowler, 2008).

1.4.4.1. Delta-aminolevulinic acid

ALAD is a metalloenzyme containing -SH groups and Zn, which are essential for its activity, but on the other hand turns this enzyme highly sensitive to heavy metals, molecular oxygen and other pro-oxidant conditions. The impairment of its activity may result in heme synthesis depression stimulating ALAS, by virtue of negative feedback control (Quintanilla-Vega et al, 1996), leading to increased production of delta-aminolevulinic acid (delta-ALA). The consequent increased in delta-ALA body burden can be detected by the presence of a considerable amount of the precursor in circulating blood and excreted urine (Gurer and Ercal, 2000; Makino et al, 2000; Schmatz et al, 2012). Delta-ALA accumulates especially in the liver and in the CNS (Onuki et al, 2004). The mechanism whereby delta-ALA is transported to the brain is uncertain, but it was suggested that delta-ALA enters through passive diffusion via the BBB. Delta-ALA is also produced in the brain (Adhikari et al, 2006) and the presence of ALAS and other enzymes of heme biosynthesis was already detected in this organ (Maines 1980). It was proposed that excessive delta-ALA brain levels play a central role in the development of neuromuscular and neuropsychiatric manifestations of inherited and acquired porphyric disarrays, such as mood disorders, aggressiveness, hallucinations, convulsions and seizures (Adhikari et al, 2006; Demasi, 1996). Indeed delta-ALA causes CNS damage and peripheral neuropathy (Ryter and Tyrrel, 2000) being demonstrated that excess of delta-ALA is involved in neuronal cell death mainly due to its pro-oxidant effects (Adhikari et al, 2006; Onuki et al, 2004; Rocha et al, 1995; Schmatz et al, 2012). At physiological pH, metal catalyzed autoxidation of delta-ALA generates a carbon centered radical (ALA•) as well as O_2^-•. The ALA• further oxidizes to the imino form of delta-ALA, producing O_2^-•, which dismutates to H_2O_2 (Ahamed and Siddiqui, 2007; Demasi, 1996; Gurer and Ercal, 2000; Reckziegel et al, 2011; Ryter and Tyrrel, 2000). Delta-ALA-generated O_2^-• also promotes Fe release from the endoplasmic reticulum which may contribute to the generation of •OH through the

Haber-Weiss reaction (Onuki et al, 2004). Also the coupled autoxidation of delta-ALA with oxyhemoglobin [Hb-Fe (II)] produces methemoglobin and H_2O_2 (Ryter and Tyrrel, 2000). Oxidative damage to brain lipids and proteins, concomitant with cerebral Fe accumulation due to delta-ALA administration to rats, are in vivo confirmations of these evidences (Demasi, 1996). Disruption of mitochondrial membrane potential and promotion of Ca (II) release from the intramitochondrial matrix, with subsequent mitochondrial damage may also occur (Adhikari et al, 2006; Onuki et al, 2004). The inhibition of Na^+, K^+ -ATPase and alterations on gamma-aminobutyric acid (GABA) and glutamate uptake or release, are additional proposed mechanisms (Demasi et al., 1996; Emanuelli, 2003; Juknat et al., 1995).

Blood ALAD is sensitive to the inhibitory action of Pb because this metal replaces the Zn cations in the active site of the enzyme. A study where the addition of Zn plus thermic treatment resulted in the recovery of diminished activity of ALAD upon Pb exposure supports this belief (Rocha et al, 1995). Another mechanism whereby Pb interferes with the ALAD catalytic activity is through the binding to it - SH groups. Also OS induced by accumulated delta-ALA is accepted as being associated with the pathophysiology of Pb poisoning (Gurer and Ercal, 2000). However, the association between low level Pb exposure and OS has not been yet systematically explored (Ahamed and Siddiqui, 2007). Even so, direct correlations between Pb blood levels and ALAD decreased activity, with subsequent increase of malondialdehyde (MDA) erythrocytic levels, an indicator of lipid peroxidation (Franco et al, 2009), has been observed among workers exposed to Pb (Jomova and Valko, 2011). ALAD is highly sensitive to the presence of As by virtue of its sulfhydryl moiety (Flora, 2011) and indeed the exposure of rats to As already leaded to observe a significantly decrease in the brain ALAD activity (42%) (Reckziegel et al, 2011). Despite statistical correlations of decreased ALAD upon As exposure were not scrutinized in an in vivo work, brain GSH levels decreased (Flora et al, 2005). No mechanistic information is available concerning Mn interference with heme synthesis.

1.4.4.2. Porphyrins

Porphyrins are formed as intermediates of heme biosynthesis (Quintanilla-Vega et al, 1996) (Fig. 1.1) and the synthesis and intracellular concentrations of porphyrins and heme are tightly regulated trough delta-ALA, PGB and also porphyrins (considered as oxidized forms of PGB) levels (Lelli et al, 2005). Such tight regulation is due to the fact that heme synthesis is a quite sensitive process. Reduced heme concentrations in nonerythroid cells causes among several adverse effects OS, impaired function of the mitochondrial respiratory chain, Fe accumulation and cell death (Krishnamurthy et al, 2007). On the other hand enzymatic disarrays affecting this metabolic pathway (porphyrias) lead to the accumulation of heme precursors that when in excess can act as toxins (Hift et al, 2011; Krishnamurthy et al, 2007).

Excessive accumulation of porphyrins is deemed to be responsible for pathological pictures affecting the CNS and the PNS (Simon and Herkes, 2011). Porphyria has been linked to the madness that affected King George III of Great Britain and the illness of Vincent van Gogh (Lin et al, 2008). The mechanism by which altered heme synthesis results in nervous dysfunction is unknown. Even so, evidence today suggests that direct toxic effects of porphyrin precursors in the nervous system as well as intracellular metabolic derangement resulting from heme deficits contribute to the observed neurologic disorders (Simon and Herkes, 2011). Presynaptic inhibition of neurotransmitters release is a proposed mechanism; the deficiency of heme in neural tissues also produces diminished energy capacity with consequent dysfunction of the energy dependent Na^+/K^+ ATPase; there is also depletion of essential substrates or cofactors arising from the heme biosynthetic pathway defect, such as pyridoxal phosphate, Zn or glycine; abnormal products derived from the porphyrin precursors, such as hemopyrrole may also have an important role (Juknat et al, 1995; Kuo et al, 2007; Lin et al, 2011; Moore, 1998). Oxidative damages can also occur since due to its lipophilic nature, excess protoporphyrins and other porphyrins may accumulate in membranes and generate ROS upon exposure to light (430-635 nm). The formation of $1O_2$ and $O_2^-\bullet$

through photochemical reactions has been reported (Krishnamurthy et al, 2007; Ryter and Tyrrell, 2000). Porphyrins may also form a porphyrin anion radical, that may substitute for $O_2^{-}\bullet$ in Fenton-type reactions or produce $O_2^{-}\bullet$ by reducing O_2 (Ryter and Tyrrell, 2000). Under these conditions, excess porphyrins can cause oxidative damage to membrane components, with proteins and lipids as the main targets of photodamage, leading eventually to cell death (Krishnamurthy et al, 2007; Ricchelli, 1995).

In Pb-treated cell cultures, porphyrin production was found to be decreased as shown by the reduced levels of inter- and intracellular porphyrins. This effect could be primarily explained by ALAD inhibition, resulting in a decreased of monopyrrole supply for porphyrin biosynthesis. An interesting outcome from this study is that Pb concentrations could produce a biphasic effect on porphyrin production (Quintanilla-Vega et al, 1996). It has been reported that As can interfere with porphyrin metabolism, with uroporphyrinogen synthase, coproporphyrinogen oxidase and FECH being affected by this metal (Shaw et al, 2012). Little information exists on the effect of Mn in heme biosynthesis, expect a few studies demonstrating that Mn can alter heme metabolism in an in vitro assay using rat brain cells (Qato and Maines, 1985).

1.5. Control of Human exposure to chemicals

In today's industrialized world it is imperative to know the safety of chemicals present in our everyday life, leading scientists and regulatory authorities to try to safeguard the health of present and future generations (Kakkar and Jaffery, 2005). In this perspective it has become increasingly important to establish environmental and occupational limits of exposure to chemicals in order to contribute to a reduced and safer exposure (Gil and Pla, 2001).

1.5.1. Environmental and Biological Exposure limits

The aim of Occupational Toxicology is precisely the control and prevention of hazards arising from workplace exposures (Nielsen and Øvrebø, 2008). Occupational exposure limits (OELs) are key tools in this field, since these standards suggests the allowable concentrations of harmful substances in the air. Nowadays the general approach used by most organizations that propose OEL includes a review of epidemiologic and toxicological information to identify potential hazards and determine suitable exposure levels (Haber and Maier, 2002). The rationale behind OELs is that if the exposure is sufficiently low, then no or acceptably small health effects will arise (Ding et al, 2011). The concept of ‘‘adverse health effect” is important in OELs setting. According to one definition adverse effects are biochemical changes, functional impairments or pathologic lesions that affect the performance of the whole organism or reduce an organism’s ability to respond to environmental changes. Another definition describes an adverse effect as an effect that causes an impairment of functional capacity, a decreased ability to compensate for additional stress and to maintain homeostasis, an enhanced susceptibility to other environmental influences, or if such impairments are likely to become manifest in the near future (Nielsen and Øvrebø, 2008). Neurotoxicity is undoubtedly included in both definitions. Focused on the concept of adverse effects, threshold limit values (TLVs) are health based established OEL values intended to be guidelines to be used by professional industrial hygienists. Actually, most of the several regulatory systems have been developed based on the American Conference of Governmental Industrial Hygienists’ (ACGIH) established TLVs (Ding et al, 2011). They are namely the TLV–TWAs (time-weighted average), which are upper control limits for each exposure day and in a controlled work environment; the long-term life-time average exposure for each individual worker has to be less than the TLV–TWA and applies to an 8-h workday and a 40-h workweek (Nielsen and Øvrebø, 2008). There are also the health-based Biological Limit Values (BLVs) for occupational exposures, established by the Japan Society for Occupational Health (JSOH), while the German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK-Commission) applies the MAK

values as well as *Biologischer Arbeitsstoff-Toleranz-Wert* (BAT) or Biological Tolerance Values. In the European Union (EU), the Scientific Committee on Occupational Exposure Limits (SCOEL) has set the Biological Exposure Index (BEI) for compounds where the OELs may not be sufficient to protect against exposures, and where biological monitoring is more appropriate to determine the body burden (Nielsen and Øvrebø, 2008). Despite the workers are usually exposed to mixtures and not to single chemical compounds to which OELs have been set, only few OELs are established for mixtures. The German MAK commission refrains from setting up procedures for evaluating effects of mixtures due to current methodological limitations. On the other hand, ACGIH approaches consider that compounds having a similar toxicological effect on the same target organ have an additive effect, in the absence of information saying the opposite. In this case, the effects of the mixture is evaluated from the hazard index of each compound {(H.I. (i) = [(Concentration of compound (i))/TLV (i)])} and the TLV of the mixture is considered exceeded if the sum of all compound's H.I. exceeds one [Sum H.I. (i) > 1]. JSOH also evaluates mixtures from the H.I. index (Nielsen and Øvrebø, 2008).

Despite the general agreement on the need of OELs, significant disparities exist in OEL values established by different organizations and concerning the methods used to derive them, even for single chemicals (Haber and Maier, 2002), as illustrated by the differences of Pb, As and Mn's values settled by different agencies (Table 1.1). Some authors go further censuring that in practice, the final values of OELs often depend, not only on health risk assessments based on scientific evidence, but also on socioeconomic factors (Ding et al, 2011). The value of harmonizing approaches (where the concept of harmonization can be distinct from standardization) is being increasingly recognized, (Haber and Maier, 2002). To date, there is at least a trend towards convergence within the EU, but still there is no obvious evidence that OELs are becoming globally harmonized around the world (Ding et al, 2011).

Table 1.1: Occupational exposure limits (OELs)

Agency	ACGIH [1]	MAK [2]	NIOSH [3]	OSHA [4]
OEL	TLV-TWA [5]	MAK [6]	REL [7]	PEL [8]
Pb	0.05 mg/m^3	0.01 mg/m^3	0.05 mg/m^3	0.05 mg/m^3
As	0.10 mg/m^3	0.10 mg/m^{3*}	0.2 mg/m$^{3\ **}$	0.10 mg/m^3
Mn	0.2 mg/m^3	0.25 mg/m^3	1 mg/m^3	5 mg/m^3

** technical exposure limit; * if less then 10 minutes

(1) American Conference of Governmental Industrial Hygienists; (2) German MAK-Commission; (3) National Institute for Occupational Safety and Health; (4) Occupational Safety and Health Administration; (5) Threshold Limit Value—Time–Weighted Average, concentration for a conventional 8-hour workday and a 40-hour workweek; (6) Maximale Arbeitsplatz-Konzentration; (7) Recommended Exposure Limit; (8) Permissible Exposure Limit.

1.6. Biomarkers as tools to control the risk of chemical exposure

The establishment and determination of OELs are certainly an important start point to access and prevent adverse health effects is workers exposed to chemicals, such as metals. However, OELs are mostly established only for single substances and refer exclusively to concentrations in the air (Nielsen and Øvrebø, 2008). These factors limits most occupational studies to the possible causative associations between the exposure to a potential causative agent and the disease outcome in terms of clinically apparent disease (Vainio, 1998), which is quite insufficient by itself.

Moreover OELs may avoid significant adverse effect in a vast majority of the exposed workers, but may not include all the workers (Nielsen and Øvrebø, 2008). Differently the determination of biological limit values (BLV) or biological exposure indexes (BEI), which represents the level of a BM that has been directly associated with (or with the lack of) a biological effect or disease (ICOH, 2010), can enable more informed risk assessment decision making (Kakkar and Jaffery, 2005). Thus, BMs are becoming increasingly important in occupational and environmental medicine (Scherer, 2005). In practice, BMs are tools that can aid in understanding the prediction, cause, diagnosis, progression, regression, or outcome of treatments (Mayeux, 2004), since can be perceived as

observable endpoints in a continuum of events leading from exposure to disease (Scherer, 2005; Vainio, 1998). This chain of events includes: 'external dose', 'internal dose' ('absorbed dose'), 'biologically effective dose' ('target dose'), 'early biological effect' and 'health effect' (Scherer, 2005). The measurement of the "internal" dose may provide more accurate assessments as regards to individual exposures. Due to the large inter-individual variability in absorption, distribution, metabolism and excretion of chemicals, or their metabolites, individual data may vary extensively as compared with the corresponding environmental exposure data (ICOH, 2010). BMs of exposure can be seen in this context, being defined as a chemical, or its metabolite, that is measured in a compartment or a fluid of an organism (ICOH, 2010; Costa and Manzo, 1995; Mayeux, 2004; Mutii, 1999). An ideal BM of exposure is chemical-specific, detectable in trace quantities, available by noninvasive samples, inexpensive to assay and quantitatively relatable to prior exposures (Costa, 1996). By its turn a BM measuring the amount of toxic or chemical in a target organ, or its surrogate, generally indicates the "biologically effective dose" (Mayeux, 2004). BMs of effect respect to other events in this chain process. They can be defined as measurable biochemical, structural, functional, behavioral or any other kind of alteration in an organism that, according to its magnitude, can be associated with an established or potential health impairment or disease (ICOH, 2010). These markers can be also obtained from human tissue and excreta with an obvious preference for noninvasive samples (Kakkar and Jaffery, 2005). An important sub-class of BMs of effect is the BMs of early disease (or early BMs of disease). This type of BM indicates early biochemical or functional alterations, which includes a wide array of biological responses, ranging from physiological adaptation to disease, and is more closely indicative of a subclinical effect or even an early reversible clinical response (ICOH, 2010). These BMs have the potential to give early warnings by flagging the early effects of exposure, thus signaling opportunities to arrest disease through timely intervention (Vainio, 1998). In addition there is another classification for BMs of effect, the BMs of potential harm. These parameters reflect physiological and morphological changes in cell and tissues as a result of the exposure and it is important to note that these changes are not necessarily coupled with changes in cell or tissue function (Mayeux, 2004; Scherer, 2005). The fact is that BM's classification is not "hermetic" and may vary with authors.

Indeed BMs have also been classified as antecedent biomarkers (identifying the risk of developing an illness), screening biomarkers (screening for sub-clinical disease), diagnostic biomarkers (recognizing overt disease), staging biomarkers (categorizing disease severity), or prognostic biomarkers (predicting future disease course, including recurrence and response to therapy, and monitoring efficacy of therapy) (Quinones and Kaddurah-Daouk, 2009). In any case a common concept underlying BMs of effect is that they should reflect early and reversible biochemical modifications that precede structural or functional damage and may also be predictive of later responses. Thus, the knowledge of the mechanism(s) that lead to ultimate toxicity is necessary and extremely important in the research of new BMs (Costa, 1996).

However, it is important to note that BMs are not a panacea for preventing all worker illnesses and injuries. The ultimate utility of BMs in occupational health and safety is the extent to which they lead to the prevention and control of occupational disease. To date the full promise of BMs for occupational safety and health has yet to be realized, because the last 30 years have focused primarily on development of technologies, validation, and elucidation of processes and procedures for molecular epidemiology, analytical chemistry and bioinformatics. Even so, during that time there have been various significant contributions of BMs to occupational health (Schulte and Hauser, 2011).

Neurological disarrays induced by exposure to neurotoxic agents are most frequently chronic progressive disorders, where traditional early warning symptoms of the development of the disease may be lacking. They may be only clinically apparent long after exposure to the initiating factor, being at this point mostly irreversible (Kakkar and Jaffery, 2005). Thus, neurotoxic injury is thought to proceed from one or more primary biochemical alterations, through eventual irreversible cytotoxicity to neurons and permanent organic lesions (Costa and Manzo, 1995). Considering this assumption, tremendous efforts have been made to identify neuropathological and biochemical BMs of neurotoxicity prompted diseases. Diagnosis could be established in earlier stages providing a valuable timely warning of the risk and enabling patients to have a chance to

get an early treatment that may curb the disease progression (Kakkar and Jaffery, 2005; Rachakonda et al, 2004). Despite a number of reviews discussing behavioral and biochemical markers of neurotoxicity published in recent years, Neurotoxicology seems to be progressing more slowly than other fields, with regard to biological monitoring and development of markers of exposure and health effects (Costa, 1996; Costa and Manzo, 1995). Until some years ago studies in neuroepidemiology most often relied on electrophysiological measurements or on neurobehavioral tests, with neurotoxic effects frequently inferred from observed behavior (Costa and Manzo, 1995). Limitations exist regarding neurobehavioral tests, such as its low specificity, making its use when alone controversial for the establishment of exposure limits. Nowadays, it is considered that using these functional tests for monitoring the effects of chemical exposure in individual workers should be avoided and should be made carefully even in groups of workers. It is becoming increasingly perceptive that studies combining several approaches, including neurophysiological, neurochemical and behavioral tests, might provide better integrated information necessary to assess health risks (ICOH, 2010). That way, molecular BMs are expected to provide a dynamic and powerful approach to comprehend the spectrum of neurological disease, with obvious applications on disease prevention, diagnosis and disease management (Mayeux, 2004). Even more important is the possibility of their use to assess subtle changes such as those occurring due to chronic low-level exposure to environmental and/or occupational chemicals (Mutii, 1999). Still, challenges and imitations exist, such as: the complexity and the singularities of the nervous system; the problems associated with the determination of the precise targets for neurotoxic action; and the inaccessibility of the nervous system for neurochemical measurements (Costa and Manzo. 1995). Prospects for significant advances in this field will surely arrive from mechanistic studies and from the search for neurochemical parameters in peripheral tissues, easily and ethically obtained in humans, that can represent a marker related with the same parameter in the nerve tissue (Costa and Manzo, 1995; Mutii, 1999). With respect to BMs of neurotoxicity induced by mixtures of metals a great amount of work is expected to be done, since despite the interaction of metals in CNS is known from some time, not much is achieved concerning the understanding of the molecular mechanisms underlying metal mixtures toxicity (Wang and Fowler, 2008).

1.6.1. Development and Validation of Biomarkers

As mentioned before, the methods to be developed for BMs determinations should be minimum invasive, analytically specific, sensitive, simple, rapid, robust and inexpensive (Giannelli et al, 2007; Mayeux, 2004; Scherer, 2005). All these requirements are sometimes difficult to assemble (Scherer, 2005) and unfortunately the cost is always a concern. If an epidemiologic study includes thousands of subjects, the cost can be quite high unless the laboratory procedure is automated and relatively simple (Mayeux, 2004). Still other limitations on BMs studies exist: 1) it is not possible to say from BM data whether the exposure was generated by occupational or non-occupational sources; 2) a BM may not be sufficiently specific for assessing exposure to a particular chemical; 3) it is difficult to relate some exposure BMs to external exposure levels and even more difficult to establish a relationship between exposure BMs and a biologic endpoint (ICOH, 2010).

To meet all these challenges BMs' validity is a crucial matter and before they can be routinely used for workers' protection, they must be previously tested through suitable studies (ICOH, 2010). Once a promising marker candidate is identified, the next step is to develop an assay with adequate analytical performance for diagnostic accuracy studies, aiming it eventual use in routine clinical practice (Füzéry et al, 2013). Thus, the development of a new BM implies the analytical and biological validation. Concerning to the analytical validation, selectivity measures the degree to which unrelated matrix components cause analytical interference; Repeatability and reproducibility, which are factors used to quantitatively express the closeness of agreement among results of measurements performed under specific conditions, are used for the precisions' evaluations (Chau et al, 2008). Additionally the analyte stability is an essential parameter (Füzéry et al, 2013). Concerning to biological validation, some authors refer three aspects of measurement validity: 1) content validity, which shows the degree to which a BM reflects the studied biological phenomenon; 2) construct validity, which pertains to other relevant characteristics of the disease or trait; and 3) criterion validity, which shows the extent to which the BM correlates with the specific disease and is usually measured by

sensitivity, specificity and predictive power (Mayeux, 2004; ICOH, 2010). In fact the accuracy (sensitivity and specificity) of a BM is one of the most important aspects to be considered. Clinical sensitivity, which is the ability of the BM to detect truly positive subjects as positive (thus avoiding false negative results), is fundamental for preventive purposes. Clinical specificity, the ability to detect truly negative subjects as negative (thus avoiding false positive results) is usually more important for diagnostic purposes (Füzéry et al, 2013). While a BM of exposure indicates the actual exposure, a BM of effect should predict the actual risk of disease, being the predictive value of an effect BM the extent to which that particular BM is capable of correctly separating subjects with a likelihood of impairment or disease from those without (ICOH, 2010; Mayeux, 2004). The use of the statistical tool receiver operator characteristic (ROC) curves allow to determine the accuracy of a BM (Mayeux, 2004) and along with other statistical approaches has contributed quite importantly to this field. Another important issue is the investigation of the distribution of the BM results in the population of interest, namely if its expression is influenced by confounding factors such as time of the day, fed/fasted, recent exercise, smoking, demographics and how much biological variation occurs within and between patients (Jenkins, 2012).

Many published articles claims to identify BMs, but only fewer have been validated for routine clinical practice. Validation requires extensive resources and multidisciplinary expertise to establish robust correlations between BMs and population health status. Moreover BMs are still not widely applied in workers who are asymptomatic for occupational diseases (Schulte and Hauser, 2011). Only highly specific and validated BMs should be used when decisions have to be made on the workers' job fitness or his/her removal from work, in order to avoid misjudgment (ICOH, 2010). To date BMs still need to fulfill the expectations that have arisen for them and to achieve this, a more holistic approach to bring them from the laboratory to the field is imperative (Schulte and Hauser, 2011).

1.6.2. Biomarkers for Pb, As and Mn

To date several BMs have been studied and proposed to access exposure, susceptibility and detect adverse health outcomes induced by Pb, As or Mn.

1.6.2.1. Biomarkers of exposure

BMs of occupational exposures are often obtained from measurement of the parent compound or its metabolites in biological samples (Nielsen and Øvrebø, 2008), being the preferred BMs those readily obtainable in body fluid(s), or excreta (ASTDR, 2007c). Concerning to metals, its detection in these biological samples is the most common indication of exposure (Phoon, 1998) with body burden generally determined from blood or urinary concentrations (Kakkar and Jaffery, 2005) and used to propose BLVs or BEIs.

Table 1.2: Biological Exposure Indices (BEIs) for Pb, As and Mn

Agency BEI	ACGIH [(1)]	MAK [(2)]	NIOSH [(3)]	OSHA [(4)]
Pb	30 µg/dL blood	70 µg/100 mL urine	60 µg/dL blood	40 µg/dL blood
As	50 ug/g creat	130 µg/L urine	35 µg /L urine	35 µg /L urine *
Mn		15 µg/L blood**		

* density of urine of 1.024; ** as a warning value

(1) American Conference of Governmental Industrial Hygienists; (2) German MAK-Commission; (3) National Institute for Occupational Safety and Health; (4) Occupational Safety and Health Administration.

Although Table 1.2 shows established values proposed for Pb, As and Mn by different agencies, these values are frequently reviewed and other BMs continue to be investigated.

The BMs of exposure to Pb used today are measurements of total Pb levels in tissues or body fluids and from these, blood Pb concentration is the most widely used parameter for general clinical use and public health surveillance (ASTDR, 2007b; Batterman et al, 2011; Fukui et al, 1999). Even so several limitations on the evaluation of Pb body burden should be taken in account. The elimination half-time of Pb in blood is approximately 30 days and therefore, Pb concentration in blood reflects mainly the exposure history of the previous few months. Furthermore, it does not necessarily reflect the larger burden and much slower elimination kinetics of Pb in bone. Indeed, the blood comprises only <2% of the total Pb burden (Casarett and Doull's, 2007). Another limitation is that blood Pb can be influenced by short-term variability in exposure, which may have only minor effects on Pb body burden (ASTDR, 2007b).

It would also be desirable if Pb in urine could be a valid substitute to blood Pb levels, as a more simple and a less invasive method. Several attempts were done to use urinary Pb as a surrogate of blood Pb (Fukui et al, 1999), with measurements of urinary Pb levels actually used to assess Pb exposure (ASTDR, 2007b). Occupational studies exist where urinary Pb correlated significantly with blood Pb, but caution is advised since: i) despite urinary Pb concentration may increase exponentially with blood Pb levels, it can exhibit relatively high intra-individual variability even at similar concentrations of Pb in the blood (ASTDR, 2007b; Moreira and Neves, 2008); ii) estimation of Pb in blood from urinary Pb can be possible on a group basis, but hardly applicable on an individual basis (Fukui et al, 1999); iii) for low level exposures urinary Pb levels are close to the detection limit of the analytical methods; iv) Pb urinary excretion reflects mainly recent exposure; v) and the determination of Pb in urine is further complicated by the decrements in kidney function, in association with nephrotoxic Pb effects (ASTDR, 2007b).

Pb can also be measured in other tissues. Pb in bone is considered a BM of cumulative exposure because the metal accumulates in the skeleton over the lifetime (Casarett and Doull's, 2007). Nevertheless, Pb in bone is an invasive and not handy method. Tooth Pb have been considered a potential BM for measuring long-term exposure (e.g., years)

because Pb that accumulates in tooth dentin and enamel appears to be retained until the tooth is shed or extracted. Since Pb is incorporated into human hair these samples has been explored as a possible noninvasive approach for estimating Pb body burden. This approach is subject to errors from contamination of the hair surface with environmental Pb and contaminants in artificial hair treatments. Hair Pb is also a relatively poor predictor of blood Pb, particularly at low concentrations (<12 μg/dL). Pb is excreted in human sweat, but it has not been widely adopted for monitoring Pb exposures since may reflect excretion of Pb in the skin that had not been absorbed into the blood. Indeed, despite Pb concentrations in sweat were already found to be elevated in Pb workers, sweat and blood Pb concentrations were poorly correlated. Interestingly, effect BMs can be used to assess exposure to Pb and include the measurement of BMs of impaired heme biosynthesis, such as blood protoporphyrin, erythrocyte ALAD activity and urinary coproporphyrin and delta-ALA (ASTDR, 2007b).

Most of the inhaled As (the most relevant path in occupational exposures) is absorbed from the lungs or the gastrointestinal tract and is excreted in the urine, mainly within 1–2 days. Thus, As is rapidly cleared from blood, which is the reason why measurements of blood As reflect recent exposures or exposures to high As levels (ASTDR, 2007a; Marchiset-Ferlay et al, 2012). Urinary As measurements have been considered more reliable than blood, because urinary elimination is the major route for the excretion of this metalloid (ASTDR, 2007a; Marchiset-Ferlay et al, 2012, Morton and Mason, 2006). Therefore, urinary As has been the most used BM in epidemiological and occupational studies as indicators of recent As exposure, by both ingestion or inhalation (Marchiset-Ferlay et al, 2012). Good correlations have been found between the concentration of As in workplace air and the concentration in the urine of workers exposed by inhalation (ASTDR, 2007a). Differently from the general view, some authors defend that in chronic and continued exposure to As by ingestion through drinking water, steady-state concentrations of As in blood and urine are achieved and that these parameters may have the potential to serve as BM of past exposure. In these studies strong correlations were obtained between the levels of As in blood and urine and also between blood As and As concentrations in the water. The authors argue that in long-term exposure situations,

blood As receives inputs not only from recent exogenous exposure but also from tissue compartments, thus reflecting the total internal As burden (Hall et al, 2006). Still respecting long term exposure speciation of urinary As may also indicate the extent of past cumulative exposure to the metalloid, more specifically by measuring the levels of monomethylarsenic (MMA) and dimethylarsenic (DMA) (ASTDR, 2007a) resulting from metabolic methylation of ingested or inhaled inorganic As (Chen et al, 2005). Opting for As speciation measurements, they provide another advantage as compared with the determination of total urinary As, since certain forms of As compounds, arsenobetaine and arsenocholine (Irvin and Irgolic, 2004) are excreted unmetabolized in urine after ingestion of certain seafoods. "Fish As" is essentially nontoxic and consequently analytical methods based on the total urinary As content may overestimate exposures to the As species that are of health concern. However, adequate methods for distinguish these compounds from other forms of As in urine, such as liquid chromatography inductively coupled plasma mass spectrometry (LC-ICPMS) or high performance liquid chromatography (HPLC) may not be available for common laboratories to be applied as a routine screening method (Chen et al, 2005). In routine procedures total values of As can be chosen as exposure BMs, although prior to collection of the samples some food precautions should be taken (Marchiset-Ferlay et al, 2012).

Pertaining to less used biological samples, As accumulates in hair and nails due to its affinity for the abundant -SH groups in keratin. Hence, As concentrations in these slow growing tissues are considered good measurements of past exposure (Hall et al, 2006). These values may increase from several- to over 100-fold following As exposure and remain elevated for 6–12 months, with reports mentioning that inhalation exposure of workers to about 0.6 μg/m^3 of As in the air significantly increased As levels in nails. However, there was wide variation between subjects and minimum exposure levels that produce measurable increases in As levels in hair and nails have not been yet precisely defined (ASTDR, 2007a).

Mn levels in biological samples such as blood, urine, feces and hair have been investigated as BM of exposure (ASTDR, 2007c). Several works describe the determination of Mn levels in the blood and urine of welders, ferroalloy smelters, Mn oxide production workers and dry-cell battery workers (Cowan, 2008). Yet the suitability of these BMs is highly controversial and what is generally achieved is that the blood and urine levels may be used to confirm exposure and possibly manganism (Phoon, 1988). However, they only indicate average levels of exposure on a group basis, such as an exposure in an occupational setting, (ASTDR, 2007c) but are not suitable to be individually used (ASTDR, 2007c; Bader et al, 1999; Cowan, 2008). A study by Lucchini et al. (1995) seems to be the only evidence that blood and urine Mn levels can be correlated with Mn exposure on an individual basis, although this correlation did not exist for cumulative exposure. Also blood Mn concentrations appear to be related to intake from food, water, and air, but once again with large individual differences (ASTDR, 2007c). Another limiting factor is the rapid rate of Mn clearance from the body, because excess Mn in blood is rapidly removed by the liver and excreted into the bile. Thus, the levels of Mn in blood has been used as an indicator of the previous few weeks of exposure, generally less than one month (ASTDR, 2007c; Batterman et al, 2011; Santamaria, 2008). Outcomes from occupational studies, also indicated that there may have a plateau level of homeostatic control of this metal (ASTDR, 2007c) and that blood Mn may be influenced by the dietary intake, which may also confound the studies' results (Santamaria, 2008). Since the urinary excretion of Mn is no more than approximately 1% and urinary Mn can only indicate exposure within the past few days, it is expected that urinary Mn may not be a sensitive indicator of Mn exposure (Batterman et al, 2011; Santamaria, 2008). Accordingly, urinary Mn already failed to allow a differentiation between exposed workers and controls (Bader et al, 1999) and no studies show a dose-response relationship between urinary Mn and health disorders (Phoon, 1988). Some authors recommend that urinary Mn should be abandoned as a BM of exposure to Mn (Cowan, 2008).

In alternative, since the feces are the primary route of Mn excretion, in theory the determination of fecal Mn concentrations should be valuable. However, there is no significant correlation between fecal excretion of Mn and occupational exposure to the

metal (ASTDR, 2007c) and the collection method truly limits its use as a BM (Cowan, 2008). Correlations between exposure levels and Mn concentration in hair were already determined (Bader et al, 1999; ASTDR, 2007c). Nevertheless, exogenous contamination may yield values that do not reflect the absorbed doses (ASTDR, 2007c).

The lack of BMs of exposure to Mn has made impossible to identify subjects who may be in danger of progressive manganism, which is aggravated by the fact that early signs and symptoms are not evident to the clinical practitioner. The search for a practically useful BM is essential to Mn neurotoxicity research (Cowan, 2008). Until then and despite all the limitations, both blood and urine Mn levels have been the most widely used BMs of exposure in occupational studies. In this respect the WHO recommends repeated screening of subjective symptoms and thorough a regularly clinical examination together with estimations of the level of Mn in these biological samples (Phoon, 1988).

External and internal exposure values of Pb, As and Mn

Table 1.3: Pb, As and Mn levels in air and biological samples

Metal	Air	Biological samples	References
Pb	0.5 μg /m^3 , EU	7 - 22 μg/100 g in blood, urban poulations	(Ec.europa.EU, 2014; EPA, 2014)
	2.7 μg/m3 , US 1985	4 - 270 μg/g creat, urban populations	
	0.5 μg/m3, US 2012		
As	<1 to 3 ng/m^3 , US remote areas	0.5 - 2 μg/L in blood and <1 μg/L in urine, not exposed	(ASTDR, 2007a; MAK commission, 2005; Marchiset-Ferlay et al,2012)
	20 - 30 ng/m^3, US urban areas	2–41 μg/L in blood upon exposure to 5–410 μg As/L in drinking water	
		71 μg/L in urine (range 10–340 μg/l), copper smelters	
		16.9 μg/g creat (range, 2.6–50.8 μg/g), occupational exposure	
		8-hour TWA As air concentrations of 48.3 μg/m3	
Mn	0.071 μg/m^3, Canada	1-8 μg/L in urine and 4–15 μg/L in blood, not exposed	(ASTDR, 2007c; Health Canada, 1979; Santamaria, 2008; Sohler et al, 1979).

Several reports concerning to concentration values of Pb, As and Mn are available, for both air and biological samples (Table 1.3). These works are important since provide references that can be used to suggest excess exposure situations.

1.6.2.2. Biomarkers of susceptibility

BMs of susceptibility can be seen as indicators of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific chemical substance (Slikker and Bowyer, 2005).

It has been known for centuries that there is large variation in susceptibility to Pb exposure, with some individuals experiencing toxic effects at levels that others can sustain without any problem. Some of these differences may have a genetic basis and namely, polymorphisms in genes for ALAD, which is the major Pb-binding protein in the blood (Bergdahl et al., 1997), has been shown to affect not only blood Pb levels but also its relationship with some toxic effects (Scinicariello et al, 2007). Indeed ALAD2 carriers have a lower risk of toxic effects than ALAD1 homozygotes at the same level of exposure (Tian et al, 2013) and some authors suggest that the effects of Pb on neurobehavioral functions tends also to be worse among ALAD1 homozygotes. Chia et al. (2004) also reported that workers with ALAD 1-1 genotypes have significantly higher urinary delta-ALA and poorer neurobehavioral scores involving motor dexterity compared with those who have delta-ALA D1-2/2 -2 genotypes.

Much of the susceptibility to As-related health effects is determined by the large interindividual variation in As methylation (Loffredo et al, 2003). The trivalent forms metabolites (particularly MMA III) are most associated with adverse health outcomes, while DMA shows the lowest retention, constituting the largest portion of metabolites in urine (60 –80%). The excreted proportion of MMA and the ratio of MMA:DMA are an accurate reflection of harmful metabolites being retained in body tissue. The distribution of urinary As metabolites is determined to a greater extend by genetic variations and is

illustrated by the aggregation of methylation patterns within families (Antonelli et al, 2014; McClintock et al, 2012).

Also Mn detoxification via pancreatic secretion and bile/urine excretion seems to vary according to an individuals' genotypic status. Mn miners experiencing the same exposure environment may have different levels of blood Mn due to inter-individual variations in genetic polymorphisms. Thus, harmful effects of Mn cannot be precisely predicted by merely elucidating the Mn levels in the exposed population. Simultaneous monitoring through the BMs of exposure, effect and susceptibility, may provide a wider view to predict the subjects that are more prone to Mn related diseases. Namely a significant association between the CYP2D6*2 polymorphism (a BM of susceptibility) and the latency of chronic Mn poisoning has been reported (Vinayagamoorthy et al, 2010).

1.6.2.3. Biomarkers of effect

The knowledge of the toxicity mechanisms of metals at tissue, cellular and molecular levels is crucial for the discovery of new BMs and also for its selection. On the basis of OS, dopaminergic, cholinergic and heme synthesis alterations induced by Pb, As or Mn, several potential effect BMs will be described.

Lead

Serum PRL has been mentioned as an indicator of the dopaminergic function, which is a target of Pb (Meeker et al, 2009). Despite not very well documented, PRL can be increased by exposure to Pb (Alessio, 2006; Roses et al, 1989). In vivo studies describe elevated serum PRL concentrations in Pb -exposed rats (Govonia et al, 1984) and Pb exposure was already inversely associated with PRL among men and pregnant women without occupational exposure, but positively associated with PRL among occupationally exposed men (Meeker et al, 2009). However, in some other studies PRL levels were not altered by Pb (ASTDR, 2007b). The effects of Pb on cholinergic functions have also been

consistently reported in the literature, with references that these alterations may precede the well-known neurobehavioral and neurophysiological end points observed in Pb neurotoxicity (Ademuyiwa et al, 2007). Accordingly, deficits in the performance on tests of spatial learning and short-term memory of Pb-exposed rodents were considered consistent with dysfunctions of cholinergic innervation (Reddy et al, 2003). It was suggested that AChE activity in the erythrocytes could serve as a peripheral surrogate dose–effect index of neurotoxicity on cholinergic function in occupational exposure to Pb (Ademuyiwa et al, 2007) and indeed, decreased AChE erythrocytic levels were observed in workers exposed to this metal (Weiss, 2006). Free radicals can induce AChE activity decrease, leading some authors to speculate that the inhibitory effect of Pb on AChE observed in the workers could be due to free radicals produced by Pb (Amal and Mona, 2009).

Pb effects in heme synthesis are also broadly known and documented (Goering and Fowler, 1987; Seth et al, 1976) being considered that many of the clinical manifestations of Pb poisoning, such as anemia and neuropathy, may be the result of altered heme biosynthesis, likewise in seen in inherited porphyrias (Moore, 1998). Over 99% of the Pb present in the blood accumulates in erythrocytes, where over 80% is bound to ALAD (Ahamed and Siddiqui, 2007). Delta-ALA and ALAD levels in blood are considered one of the most reliable indicators of Pb intoxication (Chibe at al, 1996; Reckziegel et al, 2011; Rocha et al, 1995), even with relatively low Pb blood concentrations (Reckziegel et al, 2011). However being achieved that the degree of inhibition of ALAD can be correlated with blood Pb concentrations (Thompson et al, 1977) some discussion exist regarding to Pb threshold levels. A potential Pb threshold between 3.2 and 4.8 µg/dL has been presented, above which ALAD may be inhibited by Pb and below which it may be insensitive to Pb or may even be activated (Gurer and Ercal, 2000; Wang et al, 2010). Delta-ALA levels of 3 mg/g creatinine in exposed workers were also indicated as a threshold to detect levels of Pb in blood equal to or higher than 20 µg/dl. Indeed the test results pertaining to one group of studied workers showed a sensitivity of 92% and a specificity of 90%, which was considered by the authors as relevant to validate the results (Caldeira et al, 2000). Since the inhibition of ALAD activity results in elevated delta-ALA

levels in blood, consequential increase of urinary delta-ALA excretion is expected and in fact, urinary delta-ALA have been used in clinical diagnosis of chronic occupational Pb intoxication (Wang et al, 2010). Persons working around automobile, Pb batteries and leaded gasoline show elevated delta-ALA concentrations in urine (Adaudi and Aliu, 1980). Actually, urinary delta-ALA has been used with two purposes: as a BM for Pb exposure and as marker of Pb early biologic effect (Ahamed and Siddiqui, 2007), with limits of 5 or 15 mg/L settled for workers in general (Higashikawa et al, 2000). However caution is recommended. Urinary delta-ALA levels augment with increase blood Pb when blood Pb levels are above 22.4 µg/dL and rises markedly, when blood Pb concentrations are above 35.5 µg/dL (Wang et al, 2010), suggesting a nonlinear relationship. Besides, urinary delta-ALA does not seem to be a sensitive indicator when Pb exposure is low. In other works, no relationship between urinary delta-ALA and blood Pb levels has been obtained for Pb concentrations in blood bellow 20 µg/dL (Makino et al, 2000). Further studies are necessary to examine how heme synthesis is affected by exposure of workers to Pb at low levels.

It is also largely described that patients suffering from Pb poisoning show an accumulation of porphyrins due to the inhibition by Pb of heme biosynthetic enzymes. These enzymes are ALAD, coproporphyrinogen oxidase and FECH (Ahamed and Siddiqui, 2007; Gurer and Ercal, 2000; Moore, 1998). Typically increased urinary excretion of coproporphyrin, as well as accumulation of protoporphyrin in erythrocytes is observed (Quintanilla-Vega et al, 1995). Thus, alterations in porphyrin metabolism have provided a useful means of detecting and assessing the severity of Pb exposure and poisoning (Moore, 1998).

Arsenic

There are practically no studies evaluating the effects of As exposure on PRL release. A few works document that exposure to As leads to reductions of DA content in brain areas involved in the regulation of PRL release (Bardullas et al, 2009). These outcomes are

suggestive that exposure to As could interfere with PRL levels. Recent reports mention that sodium arsenite can cause a reduction in plasma PRL levels (Jahan et al, 2012) and induces cell death in anterior pituitary cells (Ronchetti et al, 2010).

Both arsenite and arsenate can cause cholinergic dysfunctions in rats in a dose dependent manner, with concomitant AChE inhibition (Kobayashi et al, 1987; Rodríguez et al, 2003; Roy et al, 2006) and consequent decreases in cellular metabolism, deformities of cell membranes and disturbances in nervous activity (Yousef et al, 2008). Differently one study revealed a significant increase (20%) in AChE brain activity in As exposed rats, but the explanation for this different outcome was attributed, to the dose and length of the treatment that possibly triggered an hormetic response (Herrera et al, 2013).

Also, increased levels in blood of ALAS (ASTDR, 2007a) and changes in ALAD activity were observed upon exposure to both As (III) and (V) (Bhadauria and Flora, 2004) being considered that the ALAD activity values in blood could be used to estimate its enzymatic activity in the brain (Reckziegel et al, 2011).

The urinary porphyrin profile is a useful indicator for biological effect resulting from As exposure. Significant increases of uroporphyrin and coprophorphyrin in mice subchronically exposed to As (III) (sodium arsenite) or As (V) (sodium arsenate) by the same route and at concentrations of 20 and 50 mg/L were observed. Concerning this subject, and after examining other works, discrepancies were noted and explained by dissimilar lengths of exposure (Wu et al, 2004). In humans significant increases in urinary coproporphyrins were found in smelter workers exposed to arsenic trioxide dust, but the mean concentration of uroporphyrin was very similar to that of control (Krishnamohan et al, 2007). Differently, significantly increased urinary uroporphyrin and coproporphyrin levels were observed in individuals exposed to As released from a burning contaminated coal; correlations of increased uroporphyrin and coproporphyrin with higher urinary As were also determined in workers (Ng et al, 2005). Further studies are required to clarify this apparent discrepancy among human populations (Wu et al, 2004).

Manganese

The investigation of Mn effects on PRL is broadly documented although leading to controversial results. Serum PRL has been proposed as an index of Mn occupational exposure, through inhalation (Ellingson et al, 2003) and authors defend that the parameter is a possible indicator of Mn action on DA neurotransmission (Takser et al, 2004). Several positive results were obtained such as increased serum PRL levels in workers exposed to Mn (ASTDRb, 2007; Ellingson et al, 2003; Meeker et al, 2009), with dose–effect relations between PRL and Mn concentrations in blood and urine and the estimated external exposure dose (Takser et al, 2004). Namely, low level exposure to Mn oxides of workers from a ferro-manganese plant resulted in increased prevalence of abnormally high serum PRL values (Ellingsen et al, 2007), with analogous observations in Mn exposed welders (Myers et al, 2003b) and in a mining works in central Mexico (Kim et al, 2009). It is also referred that environmental exposure to Mn may also contribute to abnormally high serum PRL in the general population (Smargiassi and Mutti, 1999). On the other hand, other studies show that serum PRL levels were not increased in workers chronically exposed to airborne Mn, being impossible to relate serum PRL concentrations with atmospheric Mn exposure (Aschner, 2006; ASTDR, 2007c; Kim et al, 2007). Other unclear issues respects to a biphasic response of DA due to Mn exposure, reported in workers that differently from controls exhibited an absence of a relationship between DA and PRL. The authors speculated that this phenomenon could due to loss of normal feedback control by Mn exposure and in this view, the mechanism of Mn toxicity was presumed to be via disruption of homeostasis between dopaminergic tonic control and PRL regulation (Kim et al, 2007). Despite such complex results, mechanistic studies establish that Mn stimulates DA autoxidation in dopaminergic neurons and indirectly modulates PRL secretion, thereby leading to an increase in circulating PRL levels (ASTDR, 2007c; Marreiha dos Santos et al, 2011).

The potential of Mn to affect central cholinergic structures was not fully appreciated, since most research on manganism has been focused on the involvement of the

dopaminergic system. However some salient features of manganism, like the intensity of mood disturbances cannot be explained only by the disruption of brain dopaminergic systems. Rather, the complex clinical picture of manganism may be consistent with the effects of Mn on additional neurochemical systems, foremost the cholinergic system, known to be crucial in modulating emotional response and higher cognitive functions. Damage to the cholinergic neurons may interfere with the cholinergic–dopaminergic balance, leading to instability of mood and even to a manic-depressive psychotic state that is observed in some cases of manganism (Finkelstein et al, 2007). Miners long-term exposed to exceedingly levels of Mn may exhibit changes in the activity of blood AChE and ACh (ASTDR, 2007c) as well as RBC-AChE in other exposed workers (Finkelstein et al, 2007).

Only a few and unclear results exist regarding to Mn induced hematological effects. In one study the analysis of blood from persons chronically exposed to high Mn levels in the workplace did not reveal any significant hematological effect. Differently, in vivo studies with rats exposed to Mn through diet leaded to find significantly decreased hematocrit and hemoglobin levels (ASTDR, 2004). Even less information is available pertaining to eventual Mn effects in the heme biosynthetic pathway and one study suggested that Mn can actually interfere at this level. More specifically, Mn (II) seems to inhibit ALAS activity in liver and brain. Also few references exist concerning Mn and hematological effects: in an in vitro study the inhibition of liver and erythrocytes ALAD by Mn was observed (Maines, 1980) and in other experiment it was described that this metal can competitively inhibit ferrochelatase (FECH) (Hift et al, 2011). The content of these information points undoubtedly for the need of studying Mn effects at this level.

Cases of metals exposure, biomarkers levels and neurotoxicity

According to NIOSH, blood Pb values above 40 μg/100 g are indicative of excess exposure, with concentrations above 60 μg/100 g requiring removal from the exposure (NIOSH, 1994). ACGIH (1998) consider that elevated blood Pb concentration (>10 μg/dL) is an indication of excessive exposure in children (ASTDRc, 2007) although neurobehavioral impairment was already observed in children with Pb blood levels below 10 μg/dL (Canfield, 2003) along with IQ decrements at even lower blood Pb levels (< 5 μg/dL) (ASTDR, 2007b). According to MAK urinary As values should remain below 50 μg/L for prevention of non-carcinogenic toxic effects. Changes in the peripheral nerves of occupationally exposed persons were detected even at a mean As concentration of 71 μg/L in urine and peripheral neuropathy was observed in 51 persons with urinary As concentrations above 100 μg/L (MAK, 2002). Air Mn concentrations producing effects in chronically exposed workers may range from about 0.07 to 0.97 mg Mn/m^3, while workplace inhalation exposure levels producing overt symptoms of manganism have been documented for concentrations of 2 to 22 mg Mn/m^3 (ASTDR, 2007c). In other studies impaired visual reaction time, hand-eye coordination and hand steadiness were detected in workers exposed to concentrations of Mn dioxide in respirable dust from 0.021 to 1.32 mg Mn/m^3 (the time of exposure ranged from 0.2 to 17.7 years). Also Bast-Pettersen et al (2004) found modifications on neurobehavioral end points in a group of male workers in Mn alloy plants with 0.753 mg Mn/m^3 in inhalable dust and with Mn levels in blood and urine corresponding respectively to 189 nmol/L in blood and 3.9 nmol/mmol urine creat. In another study, Myers et al. (2003b) observed changes in several neurobehavioral end points in a group of South African Mn smelters. Their mean values were 12.5μg/L in blood and 10.5 μg/L in urine, while control workers had mean values of 6.4 μg /L for blood Mn and 0.96 μg/L for urinary Mn (ASTDR, 2007c).

1.6.3. Biomarker(s) for metal mixtures

Table 1.4 presents several BMs proposed to control Pb, As and Mn induced toxicity. However, being BMs essential tools for the control and prevention of risk of exposure to neurotoxic agents, even for single exposures most of the outcomes pertaining to Pb, As and Mn BMs are scatter and hardly permits the translation from experimental results to humans.

Table 1.4: Proposed BMs of effect in peripheral samples for Pb, As, Mn and mixtures of these elements.

Exposure	Biological Sample	Biomarker	References
Pb	Serum	PRL	Govonia et al, 1984; Meeker et al, 2009
	Blod erythrocytes	AChE	Ademuyiwa et al, 2007; Weiss, 2006
	Blood	ALA and ALAD	Chibe at al, 1996; Reckziegel et al, 2011; Rocha et al, 1995
	Urine	ALA	Adaudi and Aliu, 1980
	Blood erythrocytes	Protoporphyrin	Moore, 1998
	Urine	Coproporphyrin	Moore, 1998
As	Serum	PRL	Jahan et al, 2012
	Blood	ALA, ALAS, ALAD	ASTDR, 2007a; Bhadauria and Flora, 2004; Reckziegel et al, 2011
	Urine	Uro- and coprophophyrin	Krishnamohan et al, 2007; Ng et al, 2005; Wu et al, 2004
Mn	Serum	PRL	ASTDR, 2007c; Ellingson et al, 2003; Marreiha dos Santos et al, 2011; Meeker et al, 2009; Takser et al, 2004
	Blood and blood erythrocytes	Ach and AChE	ASTDRb, 2007; Finkelstein et al, 2007
Pb + As	Urine	ALA	Wang and Fowler, 2008; Whittaker et al, 2011
Pb+As+Mn+others	Serum	PRL	de Burbure et al, 2006

With respect to BMs of effects induced by the mixture of these metals even less progress has been achieved and in truth barely any information is available (Zhai et al, 2005), despite the knowledge that interactions among metals within organisms surely occurs. These events may happen when metals compete for binding locations on specific enzymes or receptors during the processes of absorption, excretion, sequestration or even at the target site (Fairbrother et al, 2007). These are events that certainly lead to relevant modifications on toxicokinetic and toxicodynamic processes, turning the effects different from exposure to each metal alone. Some scarce and sparse studies with mixtures of Pb, As, and/or Mn demonstrate the induction of OS in rats receiving through drinking water a mixture of eight metals, that included Pb, As and Mn, representative of groundwater contamination in different parts of India (Jadhav et al, 2007). Other works show that there is an interaction of Pb and As on the monoaminergic systems of the adult mouse, with increased DA levels observed in rats co-exposed to this binary mixture (Mejía et al, 1997). Depressed levels of DA were found in the brain of rats co-exposed to Pb and Mn (Chandra et al, 1981). Changes in the dopaminergic marker serum PRL in children were associated with the environmental exposure to a mixture of four metals that included Pb, As and Mn (de Burbure et al, 2006). Concomitantly the experimental co-exposure to Pb and As caused synergistic inhibition of ALAD and increased ALA urinary excretion as compared to a single exposure either to Pb or As (Wang and Fowler, 2008; Whittaker et al, 2011).

1.7. Predictive Toxicology, Selection and Integration of BMs

There are approximately 100 billion neurons in the human brain that orchestrate an incredibly wide range of motor and internal regulatory functions, such as body movement, balance, visual and auditory perception, pleasure, pain and thermal sensations, hormonal and metabolic regulation, as well as highly complex behaviors, such

as language, memory, learning, and executive functions. Such diverse functional output is the product of molecular events occurring in neurons (Wang and Michaelis, 2010).

Predictive toxicology is a new area of Toxicology, still .progressing and lately focused in the development of procedures capable to predict toxic effects (the output) from chemical and biological information (the input) (Helma, 2005). Due to several particularities of CNS diseases, this science field can be very promising for the application of BMs of neurotoxicity. Many neurodegenerative disorders cannot be cured at the present time. A major challenge to clinical diagnosis is the increasing recognition that a variety of these diseases can mimic each other or coexist, with each one capable of contributing to the symptomatic expression of brain failure (Shy et al, 1993). Moreover, knowing that the contribution of chemicals to neurodegeneration is relevant, the complex and diverse functions of the nervous system provides a multitude of mechanisms by which toxicants can produce injury. This situation becomes even more challenging when mixtures of neurotoxic chemicals are involved. One of the most frustrating issues in BMs discovery is when markers discovered by one research group cannot be reproduced by others. Biological variations may explain this issue, as human beings are extremely heterogeneous and because most of the studied diseases are very complex (Shy et al, 1993). Additionally if the detection of the onset of neurological diseases is the desirable situation for purposes of prevention of irreversible dysfunctions, on the other hand the earlier the marker in the progression of biological response, the less strongly it can be expected to predict later outcomes (Mutii, 1999).

1.7.1. Multivariate methodology

In face of all the presented limitations a naïve expectation is that a single BM can capture the complex process underlying a neurological illness (Quinones and Kaddurah-Daouk, 2009) most particularly when considering that each BM alone may have its own application for specific stages of disease and when exposure to mixtures of chemicals are

a contributing factor (Rachakonda et al, 2004). The detection of neurotoxicity depends in most instances on recognizing patterns of changes rather than any single abnormality (Mutii, 1999); lately it is becoming growingly mentioned that BMs can be combined into panels in order to increase their predictive power, (Robin et al, 2013) capturing more accurately a disease state and provide information valuable for diagnosis, prognosis, and treatment (Quinones and Kaddurah-Daouk, 2009; Shy et al, 2009). Consensus guidelines states that a useful BM should have a biological sensitivity and specificity of more than 80% (Ravid, 2009) and multiparameter analysis through the combination of independent markers may improve diagnostic performance over single markers that may be lacking in sensitivity and/or specificity, most particularly with respect to early detection of the disease (Etzioni et al, 2003; Prakash et al, 2010). Lately, most of the research in predictive toxicology has been devoted to the development of algorithms (procedures or formulas for solving a problem) (Helma, 2005), aiming to establish robust correlations between BMs and individual's health status. Metabolomic analysis, nowadays focused on the discovery of panels of BMs, together with informatics tools are being used to predict a multitude of disorders (Schulte and Hauser, 2011); this novel approach is being described in recent works. Some examples are the application of multivariate analysis tools to find an optimal and minimum panel of BMs to discriminate lung cancer patients from high-risk people (Flores-Fernández et al, 2012), to predict pathological staging of prostate adenocarcinoma (Etzioni et al, 2003) and to detect early stage ovarian cancer through the quantification of multiple blood-borne BM (Edgell et al, 2010). Also multivariate analysis of brain-specific proteins and neurotransmitter metabolites was already considered as helpful in the differentiation of CNS disorders (Ravid, 2009). Several authors recognize the replacing of single-molecule BM analysis by metabolomics-based multiparameter diagnostics as representing extremely promising advances toward early detection of neurological diseases, such as PD (Ahmed et al, 2010; Quinones and Kaddurah-Daouk, 2009); the evaluation of the progression AD using discriminant analysis was already performed (Da et al, 2014; List et al, 2013). The use of multivariate regression techniques also allowed also identify a distinctive signature of highly correlated metabolites in a set of four patients, three of whom had lower motor neuron disease (LMND) (Quinones and Daouk, 2009). This perspective is defended by

some authors with respect to inhaled complex mixtures (Kakkar and Jaffery, 2005; Scherer, 2005; Schulte and Hauser, 2011) and will aso be tested in this work. Metal toxicity in people exposed occupationally is a situation demanding immediate attention, with further studies on interactions of metal mixtures utilizing BM's endpoints highly warranted (Wang and Fowler, 2008). The integration of exposure and effect BMs is an approach that might contribute to detect and diagnose early metal poisoning.

Chapter 2

Aim

Today there is an increasing incidence of neurological diseases, being known that the intensification of the exposure to neurochemicals may contribute to the increase of these diseases. Metals are among its risk factors and the elements Pb, As and Mn frequently co-occurs in occupational settings being the presence of this neurotoxic mixture largely reported in mining areas (Dahtrak and Nandi, 2009). Yet there is barely no information about the levels of occupational exposure to metals and to its mixtures in Portugal, which undoubtedly justifies a demand on the realization of studies on such a sensitive matter. Suitable markers of neurotoxicity, a crucial tool to prevent the consequent irreversible and highly limitative disorders that may affect the exposed workers, are also necessary. To date several BMs have been discussed and proposed for the detection of Pb, As or Mn induced neurotoxicity, with some achieved progress, but nearly only as regards to BMs for each metal alone. However, in vivo metal interactions due to concurrent exposure are recognized events and these events occur also in the CNS. Interactions may cause modifications on disposition and toxicodynamics and consequently, lead to changes on BMs levels. It is plausible that these changes contribute to retract the infield applicability of BMs investigated so far for each metal alone, which is in fact an approach quite detached from workers "real life" situation.

The hypothesis of this project is that the exposure to the neurotoxic elements Pb, As and Mn simultaneously will originate an effect that may be different from the sum of neurotoxic effects of each metal separately. The aim of this work is to identify BMs of neurotoxicity which may be used to control and prevent the risk of chronic low levels exposure to these elements, thus may contributing to prevent the risk of neurotoxic effects in exposed populations providing a better health and quality of life. The levels of these elements in blood and urine usually used as BMs in occupational and environmental studies provide little information concerning target organs including the brain, one of the most susceptible ones. Several markers will be evaluated, with the purpose of select the ones which may have a better correlation with the exposure to the metal mixture and with its induced neurotoxicity. These BMs may avoid the installation (or progression) of neurotoxic effects by evincing the beginning of onset subtle physiological alterations. Some information about the levels of metal mixtures in exposed Portuguese workers will

also be obtained. There is the expectation that this work will bring benefits for the workers' health and for the companies and contribute for the development of Occupational Toxicology, a very important area of safety sciences, in both public health and economic terms, in particular in Portugal.

2.1. Objectives

The main objective is the selection of BMs to be applied in the control of chronic occupational exposure to the mixture of Pb, As and Mn.

The specific objectives are:

1- To evaluate the neurotoxic effects induced by the co-exposure to Pb, As and Mn in a rat model;

2- To study the interaction among As, Mn and Pb on their deposition in target organs;

3- To identify the three-metal exposure and predict the severity of neurotoxic effects, using a multibiomarker approach;

4- To translate the selected BMs on a preliminary study in a risky population of Portuguese miners co-exposed to Pb, As and Mn.

To achieve these objectives it was performed:

i) a repeated exposure in vivo assay;
ii) the application of mathematical models to the experimental data;
iii) a preliminary study in workers exposed to the mixture of the three metals applying selected BMS.

The Chapter 1 of gives a general background focused on the increase of metal emissions in the environment, and the consequent health problems. In this context, the presence of Pb, As and Mn as mixtures in occupational setting such as mines, and the neurotoxic mechanisms and effects induced by these metals are also referred. BMs as tools to control the risk of chemical exposure are described for these three elements, and multivariate methodologies through the selection and integration of biomarkers are explored as a novel alternative to control human exposure to chemical mixtures.

Chapter 2 covers the general and specific aims of the thesis.

In Chapters 3 and 4 an in vivo assay with Wistar rats is described. The rats were exposed to Pb, As or Mn and another group was co-exposed to the mixture of these 3 metals. The goal of the experiment was to study deposition interactions among the three elements (Chapter 3), evaluate neurotoxic effects (Chapter 4) and identify BMs of exposure and/or neurotoxicity in rats co-exposed to the three metals (Chapter 4). Hence the levels of As, Mn and Pb were quantified in the brain, liver, kidney, blood and urine as well as the levels of other BMs, chosen based on the known mechanism of toxicity of Pb, As or Mn, blood and brain AChE, urine and brain delta-ALA and porphyrins profile, serum PRL and urinary total porphyrins.

In Chapter 5 a new methodology for selection and combination of BMs was applied using multivariate analysis. Modeling experimental data obtained from the in vivo assay (rats exposed to Pb, As, Mn or its mixture), several studied BMs were integrated to identify the type of exposure and predict the magnitude of neurotoxic effects of each rat (respectively by discriminant and multiple regression analysis). It was hypothesized that the integration of peripheral BMs of exposure and/or effect could bring improved prediction as compared with the customary methodology of using single BMs (Chapter 4).

In Chapter 6 a preliminary study was performed in a chronically exposed population of Portuguese miners aiming to test the selected BMs (Chapters 4 and 5). According to the

information given from the mining Company this population was exposed to a mixture of the studied metals, Pb, As and Mn.

Chapter 7 includes a final discussion and conclusions and outlines the future work perspectives.

Chapter 3

Arsenic and manganese alter lead deposition in the rat

3.1. Background

In the real world, exposures to complex mixtures are the rule rather than the exception with reports on multiple exposures to toxic metals continually emerging (Scherer, 2005). It also appears that excess metal exposure may be responsible by the appearance of neurotoxic effects in several populations around the world (Wright and Baccarelli, 2009) but while much data exists concerning exposure to single elements, limited information exists to characterize the neurotoxicity resulting from exposure to chemical mixtures (Tiffany-Castiglioni et al, 2006).

Pb, As and Mn are of considerable concern to environmental and public health issues (Fairbrother et al., 2007), owing to their inherent toxicity (Dahtrak and Nandi, 2009). While Pb and Mn are metals, As is a semi-metal, but aiming simplification purposes along the text all the three elements will be referred as metals. Pb and As are present in the top ten binary combinations of contaminants in soil and water (Fay and Mumtaz 1996) with major occupational exposures reported in metal smelters (Binks et al, 2005; Mejía et al, 1997). Co-exposures to Pb and Mn have been described in children as well as in several occupational cohorts (Shukla and Singhal, 1984). The 3 metals are among the major toxicants in mining environments (Dahtrak and Nandi, 2009), with concurrent exposures from soil and dust (Reglero et al, 2009; Rodriguez, et al, 1998). Thus, considering the actual trend for Pb, As and Mn to be ubiquitous contaminants, the likelihood of co-occurrence in many other environmental settings should definitely be considered and identified.

Continued exposure to Pb, As and Mn may lead to long-term toxic effects (Dahtrak and Nandi, 2009); since these metals readily accumulate in multiple tissues (Martinez-Finley et al, 2012). Food- and water-borne Pb is distributed to several organs after gastrointestinal (GI) absorption (ASTDR, 2007b; Casarett and Doull's, 2007; Gerhardson, 1995; Hodgson, 2010) with prevailing neurological, hematological and nephrotoxic effects (El-Safty et al, 2004; Hodgson, 2010). Unabsorbed Pb is excreted

predominantly in the feces and absorbed Pb is excreted mainly in the urine (Casarett and Doull's, 2007). Approximately 80-90% of As is absorbed from the GI tract (ASTRD, 2007a), despite skin and lungs are considered relevant routes in occupational exposure (Casarett and Doull's, 2007). Arsenic, predominantly deposits in the liver (Yu, 1999), kidney and brain (Benramdane et al, 1999), triggering nephro- and neurological effects (ASTDR, 2007a); its predominant route of excretion is via urine (Hughes et al, 2011). Although Mn is an essential element, exposure to high levels may result in toxic effects (ASTDR, 2007c). Exposure to Mn by inhalation is the predominant route of exposure in occupational cohorts. Mn readily deposits in brain, with half-lives exceeding those in other organs (Casarett and Doull's, 2007). Upon ingestion, Mn is widely distributed via plasma, accumulating in mitochondria-rich tissues, such as brain, liver and kidney. High Mn levels adversely affect brain and liver function (ASTDR, 2007c). Mn is predominantly eliminated by fecal excretion (Casarett and Doull's, 2007).

The absorption, distribution, metabolism and excretion (ADME) of Pb, As and Mn is reasonably well understood but only for single metal exposures. Despite the general recognition that each mixture component may affect the toxicokinetics of the other mixture components (Simons, 1995), there is a dearth of information on the in vivo interaction between these metals (Kakkar and Jaffery, 2005), justifying the need to study deposition interactions and consequent modifications arising from resulting different toxicodynamics. Moreover, given the existence of common target organs of each component of the mixture, such as the brain, potential additive or synergic effects in sensitive health endpoints are a matter to deal with serious attention.

Pb and As are non-essential metals for biological functions (Al-Attar et al, 2011). In contrast, given its essentiality, a range of biochemical mechanisms (membrane transporters) regulate Mn's uptake and homeostasis (Pittman, 2005). Essential metal transporters can be "hijacked" by non-essential metals possessing physicochemical similarities (Martinez-Finley et al, 2012). Accordingly, competition between metals within a mixture may take place at relevant surface transporters, modifying their

uptake and accumulation (Spurgeon et al, 2010). In addition, once within the cells, a particular metal may occupy abundant binding sites (Kalia et al, 1984) on metalloproteins or target molecules, modifying in turn the compartmentalization of other metals, thus leading to aberrant binding and toxicity (Spurgeon et al, 2010). A given metal may also induce overexpression of transporters and/or binding proteins that alter the uptake of other metals (Kalia et al, 1984; Molina et al, 2011). These events may modify the uptake and/or retention of metals in cells, affecting their toxicokinetics profile and toxicity (ASTDR, 2000; Creton et al, 2009; Molina et al, 2011).

Since humans are more frequently exposed to toxic mixtures rather than to a single element, it is essential to understand how chronic low-level co-exposures modify metal deposition and toxicity (ASTDR, 2000). In vivo studies can serve important functions such as, the identification of adverse effects induced by chemicals and methods for biological monitoring of exposure and early adverse health effects. The generation of data from experimental studies to predict health effects in humans is a central issue on experimental toxicology (Casarett and Doull's, 2007). The present study was designed to test the hypothesis that tissue Pb levels are modified by co-treatments with As and Mn, accounting for exacerbated behavioral deficits ascertained in the metal mixture-treated rats (Chapter 4). Therefore, a sub-acute in vivo study was conducted to determine changes in Pb levels in various organs and fluids in rats exposed to Pb, As and Mn alone, and in rats treated with a mixture of these metals.

3.2. Experimental procedure

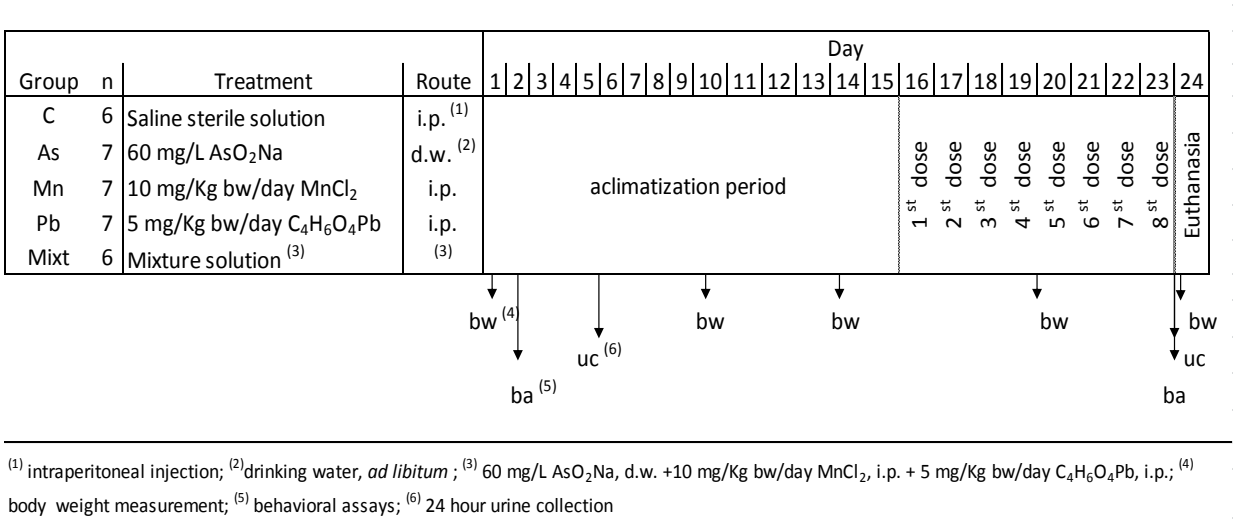
3.2.1. Chemicals

Chemicals were obtained from the following sources: Manganese (Mn) standard for Graphite Furnace Atomic Absorption Spectrometry (GFAAS) from Fluka; arsenic (As) standard solution for AAS (H_3AsO_4), di-sodium hydrogen phosphate p.a. (Na_2HPO_4; ≥

99%), hydrogen peroxide 30% (H_2O_2), magnesium matrix modifier for GFAAS [$Mg(NO_3)_2 \cdot 6H_2O$], nitric acid 65% suprapure (HNO_3) and hydrochloric acid for ultra-trace analysis (HCl) from Merck; lead acetate trihydrate puriss. [Pb $(CH_3CO_2)_2 \bullet 3H_2O$], lead for AAS standard solution, manganese chloride tetrahydrate ($MnCl_2 \bullet 4H_2O$; 99.99%), sodium (meta)arsenite purum (AsO_2Na; ≥ 99%), bovine serum albumin (>98%) and Bradford reagent from Sigma.

3.2.2. In vivo study

A repeated exposure assay was performed using male Wistar rats purchased from Charles River Laboratories ®, Barcelona, and weighting 165 –206 g. All experiments were carried out in accordance to criteria outlined in the guiding principles of the European Community Council Directive (89/609/EEC) for the care and use of laboratory animals. The rats were housed in an independent room with controlled temperature, humidity and a 12-h light/dark cycle. Their general condition was checked on a daily basis. All the animals had free access to water and food, supplied as pellets and adequate for normal growth, reproduction and maintenance. After an acclimatization period of 15 days, the animals were randomly assigned in 5 groups and the treatments were done according to the experimental design described in Fig.3.1. The administrated doses were chosen based on previous reports showing that rodents exhibited behavioral alterations (Reddy et al, 2003; Rodríguez et al, 2010; Vezér et al, 2005). During the experiment body weight was measured, behavioral assays performed through the determination of ambulation and rearing counts and 24h urine was collected on ice, using metabolic cages. After centrifugation (2500 rpm, 15') the urine samples were stored at – 80^{o}C. Prior to sacrifice (24 h after the last dose), rats were anesthetized with pentobarbital (20 mg/kg, i.p.) and blood was collected by cardiac puncture and divided into two tubes, one for whole blood and the other for serum, which was obtained by centrifugation. Upon sacrifice, brains, kidneys and livers were immediately dissected out and all the samples were stored at -80ºC.



(1) intraperitoneal injection; (2)drinking water, *ad libitum* ; (3) 60 mg/L AsO_2Na, d.w. +10 mg/Kg bw/day $MnCl_2$, i.p. + 5 mg/Kg bw/day $C_4H_6O_4Pb$, i.p.; (4) body weight measurement; (5) behavioral assays; (6) 24 hour urine collection

Fig.3.1: Experimental design of the in vivo assay.

3.2.3. Analyses of Pb, As and Mn

3.2.3.1. Sample preparation

a- Urine (2.5 mL) was digested with 1.25 mL of an acid mixture of 1:1 (v/v) 65% HNO_3: HCl at 100°C for 30 minutes, in a water bath; b- 80 mg of renal cortex (after removing the external kidney membranes) was dried-out for 5 hours at 90°C prior to digestion with 1.5 mL of 65% HNO_3 at 100°C for 6 hours in a dry heater block; c- 100 mg of blood and 80 mg of liver or brain samples were digested by Microwave-Assisted acid digestion (900 W, 30'') using Parr Microwave Acid Digestion Bombs® with 2.9 mL of oxidizing acid mixture containing 4:1 (v/v) HNO_3 65% suprapure: H_2O_2 30%. The acid digested solutions were transferred to volumetric flasks (25 mL) and the volume supplemented with deionized water and kept at 4°C until analysis.

All the glassware and sample cups for GFAAS were decontaminated from vestigial metals for at least 24 h in a 15% HNO_3 (v/v) solution, rinsed twice with distilled water and then twice with deionized water.

3.2.3.2. Atomic absorption spectrophotometry

Blood, kidney, brain, liver and urinary Pb, As and Mn concentrations were determined by graphite furnace atomic absorption spectrophotometry (GFAAS) (PerkinElmer AAnalyst™ 700) equipped with a Graphite Furnace, a programmable sample dispenser (AS 800 Auto Sampler) and WinLab 32 for AA software. Daily calibration curves for each element were obtained with standard solutions of Pb, As and Mn and with $Mg(NO_3)_2$ (0.84 mol/L) used as a chemical modifier, which was added to blanks, standards and samples in equal volumes. The quantification limits (QLs) were 3.8 µg Pb/L, 22.5 µg As/L and 5.2 µg Mn/L.

Results are expressed as µg Pb, As or Mn per g tissue (for blood, liver and kidney), per g brain protein, and as mg Pb, As or Mn per g of urinary creatinine (creat). Urinary levels of creatinine were determined by a colorimetric method with a Randox (CR510) commercial kit and brain protein contents according to Bradford protein assay (1976) using bovine serum albumin as the standard.

3.2.4. Statistical Analysis

Statistical analysis was performed with the SPSS 16.0 statistical package for Windows (SPSS, Inc., Chicago, IL, USA). Data are expressed as means ± standard deviations (SD). All the parameters were compared by one-way analysis of variance (ANOVA) followed by post hoc Tuckey's test to assess differences between groups. ANOVA for repeated measures and post-hoc Tuckey's tests were performed to compare body weight increments during the experiment among groups; Pearson correlations were determined in the co-treated group to assess relationships among Pb levels changes in metabolism and excretion organs and the accumulation of the metal in the brain. Two-way ANOVA was also performed to test the interactions between Pb, As and Mn. The significance of the results was considered when p values were less than 0.05.

3.3. Results

3.3.1. Body weight

Table 3.1: Body weight's determination in the acclimatization period (A), before the administration of the doses (PD) and after the administration of the 4th and the 8th (last) dose in controls (C), As, Mn, Pb and As/Mn/Pb mixture treated groups. Data are expressed as mean ± sd. ANOVA for repeated measures and post-hoc Tuckey tests were performed to compare body weight increments during the experiment among groups: * is p<0.05 versus the C group.

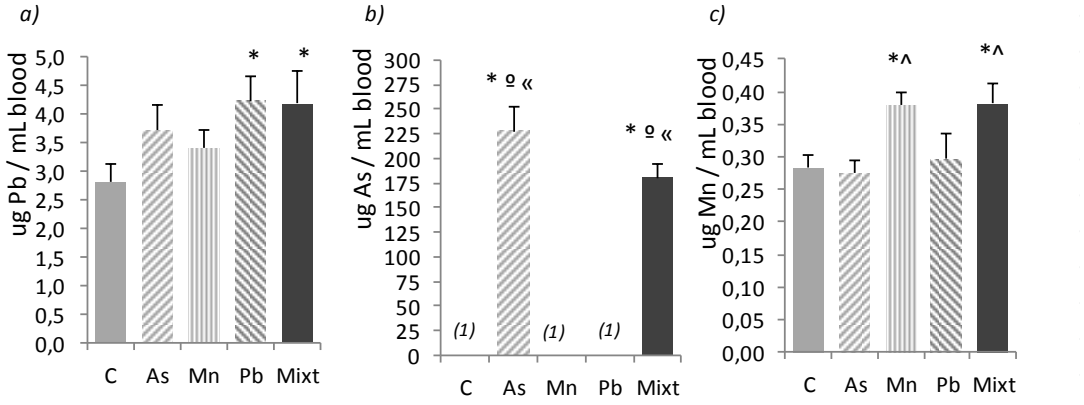
Group	A	PD	4th dose	8th dose	
C	190 ± 5	259 ± 4	289 ± 7	300 ± 9	
As	186 ± 7	251 ± 19	244 ± 18	242 ± 19	
Mn	189 ±7	248 ± 8	229 ± 8	221 ± 8	*
Pb	191 ± 4	256 ± 11	252 ± 12	255 ± 15	
Mixt	181 ± 2	246 ± 10	234 ± 14	221 ± 19	*

During the experiment the body weight was measured during the acclimatization period, in the beginning, after the administration of the 4th dose and at the end of the experiment. During the acclimatization period all the animals increased their body weight. The body weight of the Mn and Mixt treated groups measured along the total period of the experiment, revealed to be statistically different from C group ($p < 0.05$) and this difference was statistically associated with the treatments ($F= 22.9$; $p < 0.05$) (Table 3.1). In fact, in both treated groups, a body weight decrease was noted when the treatments began, while in control group an increase was observed until the end of the experiment. It should also be noticed that in spite of the lack of statistical

significance, during the administration period, the body weight of the groups exposed to Pb or As stopped to increase and became stationary (Table 3.1).

3.3.2. Pb, As and Mn levels

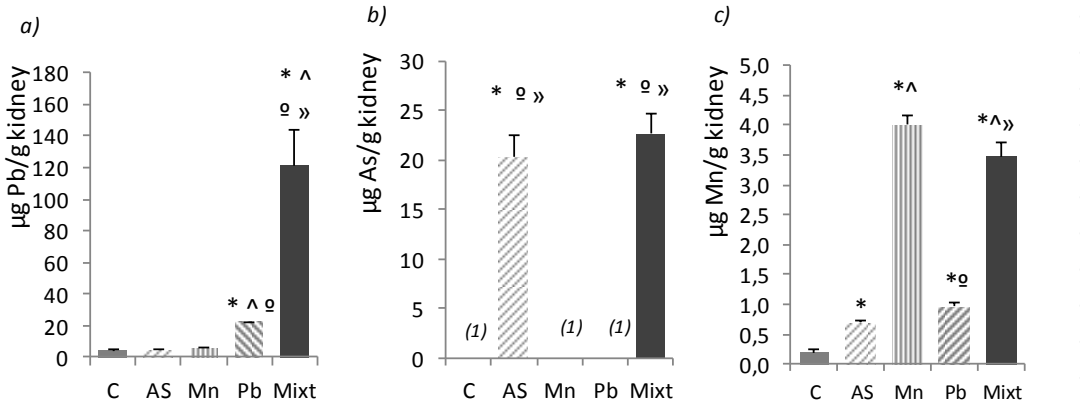
Blood



Figs.3.2. (a), (b) and (c): Blood concentrations of Pb (a), As (b) and Mn (c) of C and As, Mn, Pb and Pb/As/Mn mixture treated groups. N=6 each group. Data represent the mean ± SD. (1) In C, Mn and Pb groups, As levels were not quantified since were lower than the QL. All the groups were compared by ANOVA and post hoc Tuckey's tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

Pb, As and Mn concentrations were determined in the blood. All the 3 metals were significantly ($p<0.05$) increased in the respective single metal treated group as well as in the metal mixture-treated rats, when compared with controls (Fig.3.2a, b and c). In addition, the levels of As, in the As and mixture treated groups were significantly ($p<0.05$) higher than in the other single exposed groups (Fig.3.2b) and the levels of Mn in Mn and mixture treated groups exhibited a significant ($p<0.05$) augment as compared with the group treated with As (Fig.3.2c).

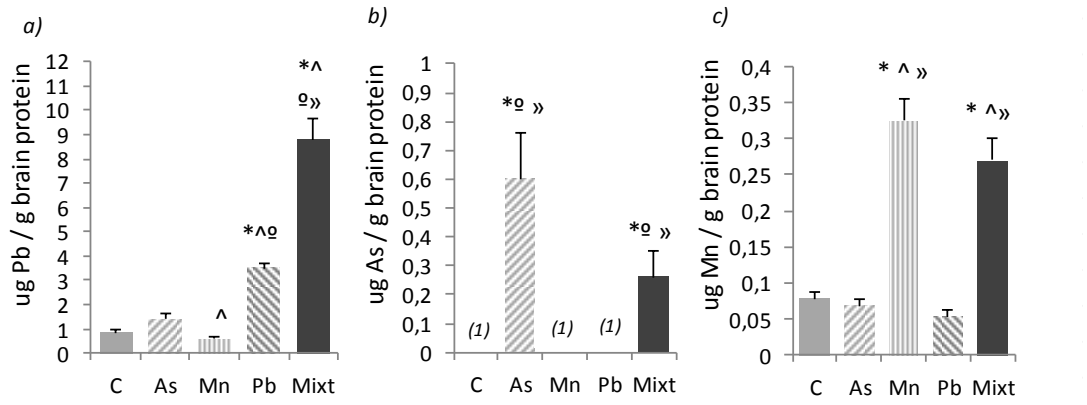
Kidney



Figs.3.3. (a), (b) and (c): Kidney concentrations of Pb (a), As (b) and Mn (c) in C, As, Mn, Pb and Pb/As/Mn mixture treated groups. N=6 each group. Data represent the mean ± SD. (1) In C, Mn and Pb groups, As levels were not quantified since were lower than the QL. All the groups were compared by ANOVA and post hoc Tuckey's tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

Kidney Pb levels in the Pb- and metal mixture-treated groups were significantly higher compared to all the other groups. In addition, Pb concentrations in the metal mixture-treated animals were significantly higher than the Pb treated group ($p<0.05$)(Fig.3.3a). Kidney As concentrations were analogous in the As- and the metal mixture-treated groups and significantly higher compared to the other groups ($p<0.05$)(Fig.3.3b). Kidney Mn levels were significantly ($p<0.05$) higher in the Mn- and metal mixture-treated groups compared to the control, As-, and Pb-treated groups (Fig.3.3c). In addition, kidney Mn concentrations in the As- and Pb-treated rats were significantly higher than in the controls (Fig.3.3c).

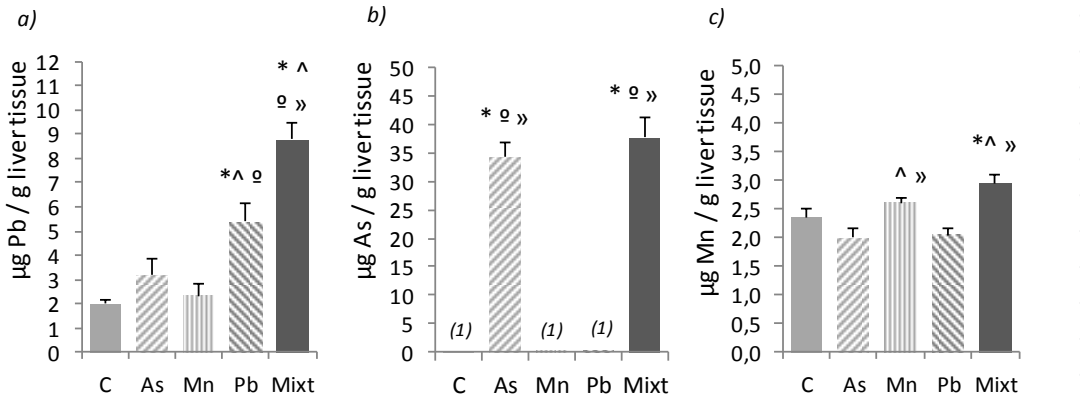
Brain



Figs.3.4. (a), (b) and (c): Brain concentrations of Pb (a), As (b) and Mn (c) in C, As, Mn, Pb and Pb/As/Mn mixture treated groups. N=6 each group. Data represent the mean ± SD. (1) In C, Mn and Pb groups, As levels were not quantified since were lower than the QL. All the groups were compared by ANOVA and post hoc Tuckey's tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

Brain Pb levels were significantly higher in the Pb- and metal mixture-treated animals compared to all the other groups ($p<0.05$). Additionally, brain Pb levels in the metal mixture-treated group were significantly higher than in the Pb treated rats ($p<0.05$) (Fig.3.4a). Arsenic levels in the As- and metal mixture-treated rats were significantly higher compared to all the other groups ($p<0.05$) (Fig.3.4b). Brain Mn levels were significantly higher ($p<0.05$) in the Mn- and metal mixture-treated animals compared to control, As- and Pb-treated groups ($p<0.05$) (Fig.3.4c).

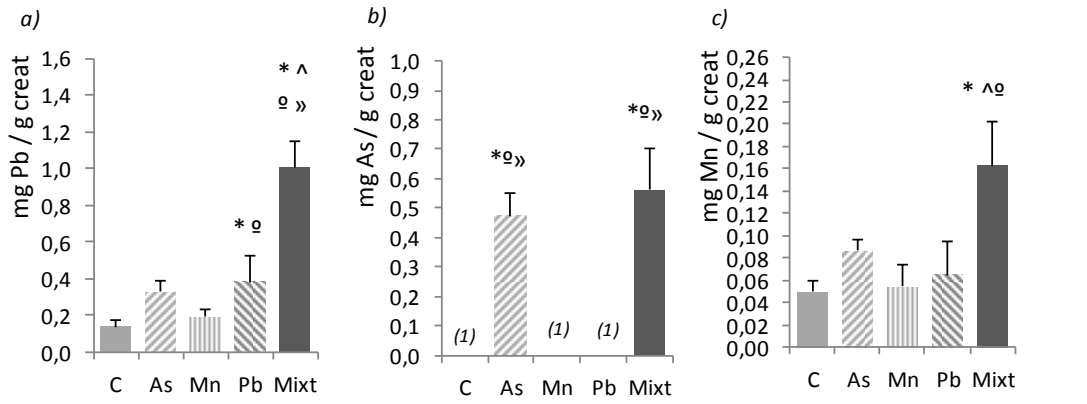
Liver



Figs.3.5. (a), (b) and (c): Liver concentrations of Pb (a), As (b) and Mn (c) in C, As, Mn, Pb and Pb/As/Mn mixture treated groups. N=6 each group. Data represent the mean ± SD. (1) In C, Mn and Pb groups, As levels were not quantified since were lower than the QL. All the groups were compared by ANOVA and post hoc Tuckey's tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

Liver Pb concentrations were significantly ($p<0.05$) higher in the Pb- and mixture-treated rats compared to all the other groups (Fig.3.5a). Moreover, liver Pb levels in the mixture-treated group were significantly higher than in the Pb treated group ($p<0.05$) (Fig.3.5a). Arsenic liver concentrations in the As- and in the metal mixture treated rats were significantly higher than in all the other groups, although there was no difference between the two groups ($p>0.05$) (Fig.3.5b). Liver Mn concentrations in the metal mixture-treated group were significantly ($p<0.05$) higher compared to the other groups, except the Mn-treated group, where Mn levels were significantly higher compared to the As- and Pb-treated groups (Fig.3.5c).

Urine



Figs.3.6 (a), (b) and (c): Urinary concentrations of Pb (a), As (b) and Mn (c) in C, As, Mn, Pb and Pb/As/Mn mixture treated groups. N=6 each group. Data represent the mean ± SD. (1) In C, Mn and Pb groups, As levels were not quantified since were lower than the QL. All the groups were compared by ANOVA and post hoc Tuckey's tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

In the metal mixture-treated rats the urinary Pb levels were significantly ($p<0.05$) higher than all the other groups and urinary Pb levels in the Pb-treated group were significantly higher compared to the Mn-treated and control groups (Fig.3.6a). Urinary As levels in the As- and metal mixture-treated rats were significantly higher compared to the other groups ($p<0.05$) (Fig.3.6b). Urinary Mn concentrations in the metal mixture-treated rats were significantly higher compared to the other groups ($p<0.05$) (Fig.3.6c), except the Pb-treated group. No significant differences were noted for urinary Mn levels between the single metal-treated groups and controls.

A significant ($p<0.05$) Pearson correlation ($r=0.827$) was determined between Pb kidney and brain levels and also between Pb liver and brain concentrations ($r= 0.902$).

Two-way ANOVA was performed to access interactions between Mn and Pb and between As and Pb in the blood, liver, kidney and brain of the mixture treated group. Statistically significant ($p<0.05$) interactions were found in all the tissues, except between Pb and Mn and Pb and As in the blood and between Pb and As in the kidney.

3.4. Discussion

Multi-exposure to metals is ubiquitous necessitating the assessment of combined potential health risks (ASTDR, 2000; Feron et al, 1995). Indeed, the additive or synergistic effects of mixtures of metals may result in the modulation of the total accumulated internal dose and changes in the compartmentalization of their components (Spurgeon et al, 2010). According to this point of view we considered that: i) Pb is a leading toxic agent in the environment (Migliore and Coppedè, 2009); ii) Pb co-occurs with As and Mn in several environmental settings (Reglero et al, 2009; Rodriguez et al, 1998); iii) a major risk of Pb exposure is its toxicity to the nervous system (Casarett and Doull's, 2013; Hodgson, 2010) and iv) As and Mn are also neurotoxic (ASTDR, 2007a and c). Thus, the assessment of in vivo interactions of As and Mn with Pb, can provide important insights for the identification of potential sensitive endpoints resulting from the exposure to this mixture and possibly clarify the origin of its increased neurotoxicity. We hypothesized that the interactions of As and Mn with Pb would lead to changes in their deposition in the brain and other tissues involved in the deposition process. Therefore, an in vivo assay was performed with Wistar rats single exposed to Pb, As or Mn and co-exposed to their mixture; the levels of each element were determined in blood, liver, kidney, brain and urine. This work revealed that the exposure to the mixture resulted in an increase of Pb concentrations in all the analyzed organs that was higher than after exposure to Pb alone, establishing

that the exposure to the mixture of Pb, As and Mn induced changes in the deposition of Pb.

3.4.1. Body weight

Body weight was measured during the experiment, which allowed the detection that all the animals had a progressive weight gain during the acclimatization period. During the treatments only the control rats continued to increase their body weight (Table 3.1), while both Pb and As treated groups exhibited a trend to body weight become stationary (Table 3.1). These results are in agreement with previous studies where the treatment of rats with similar accumulated doses of lead acetate, 25 mg/kg b.w. for 3 days, leaded to conclude that Pb exposure did not affect the animals' weight (Whittaker et al, 2010); other studies with animals treated with sodium arsenite 3 mg/kg b.w. for 12 weeks did not reduce significantly the body weight (ASTDR, 2007a). The most relevant result is concerning to Mn and mixture exposed groups, as in these groups there was a significant decrease of the body weights as compared with the controls, which was besides dose-dependent (Table 3.1). The results respecting to the Mn exposed group are in compliance with other studies where decreased body weight gains were observed in Mn exposed rats (Santos, 2008). Regarding to the mixture exposed group an exacerbated body weight decrease would be expected, but the decrease observed in these rats was actually similar to the observed in the Mn exposed group (Table 3.1). This observation leads to suggest that Mn is the major metal responsible for the body weight decrease in the co-exposed group, although by reasons that demand further explanation.

3.4.2. Metal levels

3.4.2.1. Single exposed rats

In the group exposed to Pb alone the levels of the metal significantly increased ($p<0.05$) in all the analyzed samples, when compared with the controls (Figs.3.2a, 3.3a, 3.4a, 3.5a and 3.6a). These are expected results since Pb is known to be distributed to several organs, including liver brain and kidney, through the bloodstream, and urinary excretion constitutes an important route for Pb elimination (Casarett and Doull's, 2007; Gerhardsson et al, 1995). In the same way As levels were significantly augmented ($p<0.05$) in all the biological samples of the As exposed group, relative to the controls (Figs.3.2a, 3.3b, 3.4b, 3.5b and 3.6b), which is also expected since As is transported via blood to other tissues (Jemiola-Rzeminska et al, 2007). Actually, being the liver a major site for methylation of inorganic As (Casarett and Doull's, 2007) although the kidney is a representative organ for its biotransformation and elimination (Peraza et al, 2003), As tends to accumulate in these primary target organs (ASTDR, 2007a) (Figs.3.3b and 3.5b); its accumulation in the brain (Fig.3.4) was also previously documented (ASTDR, 2007a). Urinary elimination is the major route for As excretion (De Vizcaya-Ruiza et al, 2009) which is also accordingly with our results, (Fig 3.6b). Additionally, significant ($p<0.05$) increased levels of Mn were found in the tissues of the Mn exposed group, except for the liver, (Figs.3.2c, 3.3c, 3.4c and 3.5c) as compared with controls. In agreement upon circulation in the blood, Mn is distributed largely to the organs rich in mitochondria, with the liver, kidneys and also the brain are among the tissues that mostly accumulate this metal (Casarett and Doull's, 2007). However, when Mn go into blood after i.p. administration (Fig. 3.1), it does not enter in the liver for metabolization before reaching other organs (Leggett et al, 2011), which may partially explain why Mn liver levels of the Mn treated group only slightly increased and lacked statistical significance (Fig.3.5c). This liver bypass can modify Mn disposition, increasing its accumulation in other organs, such as the brain (Casarett and Doull´s, 2007; Leggett et al, 2011) and very possibly the kidney (Figs. 3.3c and 3.5c).

Less than 1% of systemic Mn is eliminated via urine (Casarett and Doull's, 2007) explaining the analogous urinary Mn concentrations found in controls and Mn exposed rats (Fig.3.6c).

3.4.2.2. Rats co-exposed to Pb, As and Mn

Blood

In the co-exposed group the blood concentrations of the 3 elements were significantly ($p<0.05$) higher than controls, but similar to each single treated group (Figs.3.2a, b and c). It is plausible that no relevant interactions occurred among these elements during absorption, or distribution, otherwise differences in their levels would be detected in blood. With respect to the absence of interactions between Pb and Mn, over 90% of Pb and about 66% Mn in the bloodstream is bound to red blood cells (RBC) (Sakai et al, 1982). However, while the passage through Ca (II) channels is implicated in the uptake of Pb (Yokel et al, 2006), Mn uptake mainly occurs by non saturable passive diffusion (Weed and Rothstein, 1960). Moreover, once inside the RBCs, Pb primarily binds to ALAD, a saturable substrate, and only secondarily to hemoglobin, whereas Mn mainly binds to hemoglobin (Kumar and Rochester, 2008; Sakai et al, 1982). It is plausible that most Pb bounded to ALAD leaving hemoglobin available for Mn and thus, the administrated doses were insufficient to trigger interactions between Pb and Mn in the blood (Fig.3.2b). RBCs are also target cells for As compounds, although As mainly enters into these cells via a different transporter, the glucose transporter GLUT1 (Liu et al, 2006). Though, once inside the RBCs, As predominantly binds to hemoglobin (Kalia et al, 2007) and also to ALAD (Kalia et al, 2007) and indeed, despite not significant, a decrease was observed in As blood levels of the co-treated group as compared with the As treated group (Fig.3.2b).

Target organs

The levels of Pb in the liver, kidney and brain of the co-treated group significantly increased ($p<0.05$) when compared with controls and with all the single treated groups, including the single Pb treated group (Figs.3.3a, 3.4a and 3.5a). From these results, it is reasonable to assume that the exposure to the mixture increased the availability of Pb for these organs. The Pb content augments in the co-treated rats were the highest in the kidney (5.4 fold), followed by the brain (2.5 fold), while the liver had the lowest increase (1.6 fold), (Figs.3.3a, 3.4a and 3.5a). These outcomes should be carefully considered when bearing in mind the association of nephro- and neurotoxicity with the levels of Pb in these tissues in a dose dependent manner (Baranowska-Bosiacka et al, 2012) and the recognition of CNS toxicity as a particularly sensitive endpoint (ASTDR, 2007b). Probable interactions might have occurred between Pb and Mn in brain and kidney, where the augments of the Pb accumulation in co-treated rats were accompanied by a slight, though not statistically significant, decrease of Mn (Figs.3.3c and 3.4c). Several putative mechanisms are proposed to explain the uptake of Pb in target cells, like the participation of the divalent cation metal transporter (DMT1) (Martinez-Finley et al, 2012), Ca (II) channels (Charlet et al, 2012) and anion exchangers (Lind et al, 2009). DMT1 is also one of the most known mediators in the transport of Mn across membranes and Ca (II) channels are referred as well, as involved in its transport (Aschner, 2006; Martinez-Finley et al 2012; Zhu et al, 2013). Besides, these transporters are present in the kidney, in the BBB and in brain cells (Aschner, 2006; Martinez-Finley et al, 2012; Sanders et al, 2009; Wang et al, 2009). We raise the possibility that Pb and Mn competed for DMT1 and/or Ca (II) channels in these organs and that Pb entered in the kidney and brain cells in higher amounts than Mn, due to an eventual higher affinity for both transporters. With respect to Ca (II) channels Pb´s molecular similarity with Ca is broadly described (Casarett and Doull´s, 2007; Flora et al, 2011). It is also known that a significant increase of brain Pb levels appear to be influenced by DMT1 up regulation induced by Mn changes in Fe metabolism (Molina et al, 2011). We posit that an overexpression of

the DMT1 transporter, possibly triggered by Mn might have leaded to increase the uptake of Pb by brain and possibly also by kidney cells (Figs.3.3a and c and 3.4a and c).

An additional mechanism that might explain our results is the increased retention and intracellular bioavailability of low doses of Pb in major target organs that is largely determined by its complexation with a group of inducible low molecular weight glycoproteins, which are inducible by Pb exposure (Gonick, 2011). The kidney is particularly rich in these compounds (Gonick, 2011; Smith et al, 1998) and also in the brain the co-administration of Pb and Mn can result in altered affinity of Pb binding proteins accounting for increased retention of Pb in brain cells. It should be noted however that Pb binding proteins can sequester Pb in a nontoxic form and afford some protection against its toxicity, but only until certain limit (Gonick, 2011). We noted that the ratio "kidney Pb levels: urinary Pb" of the mixture treated rats was twice the ratio calculated for the Pb single treated group (respectively, 121 vs. 58.7). This higher retention of Pb in the co-treated rats' kidney may once again and according to Hodgson et al. (2007), suggest nephrotoxicity. This same view may support as well our observation of increased behavioral toxicity of the co-exposed rats (Chapter 4).

No alterations were seen in the kidney As levels of As exposed group when compared with the co-treated rats. Differently, in the brain we determined significant interactions between As and Pb ($p<0.05$) and As concentrations of the co-treated group significantly decreased ($p<0.05$) when compared with the group exposed to As alone (Fig.3.3b). Since GLUT1 is the possible major pathway uptake of As in the BBB (Liu et al, 2006) As interaction with Pb seems to have occurred during the binding of these elements inside brain cells. Metallothioneins are another type of metal binding proteins, where Zn is the dominating metal ion (Nordberg and Nordberg, 2009). Studies on the ability of metals to displace Zn from Zn-MT demonstrated that among Pb, As and Mn the highest capacity was found for Pb, As had a limited ability to displace Zn from MT and Mn had no effect on Zn displacement, at least in the liver (Waalkes et al, 1984). Concerning specifically to the brain, MT-3 is the form of MT

present in this tissue (Nordberg and Nordberg, 2009) and the confirmation that Pb can displace Zn on brain MT waits for further studies. Even so we raise the possibility that Pb occupied more MT sites then As leading to changed levels of both metals in this organ.

The levels of Pb in the liver of the co-treated group were significantly increased ($p<0.05$) when compared with all the single treated groups (Fig.3.5a) while no relevant changes were observed in the levels of Mn or As in this organ in the co-treated group (Figs.3.5b and 3.5c). Also significant interactions ($p<0.05$) were determined between Pb and As or Mn. With respect to Mn, we believe that the induction of increased expression of DMT1 contributed to increase the levels of Pb in the liver. Concerning to As, both arsenite and Pb acetate can enhance Zn-MT accumulation in liver (Albores et al, 1992; Yu et al, 2009) and since Pb´s affinity for Zn-MT in the liver is higher than As (Waalkes et al, 1984) it is plausible that the presence of As enhanced Pb accumulation in the organ. Despite a significant increase of Pb ($p<0.05$), the liver was the organ where this increase was the lowest (1.6 fold) (Fig.3.5a), possibly due to the low levels of Pb binding proteins in the liver (Gonick, 2011) and increased delivery of Pb to the kidney for excretion (Fig. 3.5a).

The significant ($p<0.05$) correlations determined between liver or kidney Pb levels and the accumulation of Pb in the brain is suggestive that a possible deficient metabolization and excretion of the metal contributed to increase its levels in the CNS.

Excretion

A significant ($p<0.05$) increase on Pb excretion was observed in the mixture exposed rats when compared to all the other groups (Fig.3.6a). The general trend for higher accumulation of Pb in the organs of the co-treated rats might result in higher amounts of Pb in the urine, since it is established that Pb urinary levels are suitable to reflect Pb body burden (ASTDR, 2007b). Despite increased Mn excretion was attained in the co-

treated group, the augment was not statistically different from Mn treated rats (Fig.3.6c). Actually urinary excretion is not the main route for Mn elimination (Casarett and Doull's, 2007) and also the mechanisms that assure a tighter regulation of the levels of this essential metal may explain the results. No relevant increased urinary excretion of As was observed due to co-exposure (Fig.3.6b). This is an interesting result, considering that urine is the predominant route for As elimination. The major fraction of excreted As is present as a GSH conjugate (Leslie, 2012). Although transport of a GSH–Pb complex has yet to be directly demonstrated, such complex may represent a transportable form of Pb (Charlet et al, 2012). Furthermore, Pb (as well as As and Mn) can deplete GSH (Erikson et al, 2004; García-Fernandez et al, 2002). Should GSH-Pb complexation represent a predominant route for Pb excretion, it is plausible that the markedly increased Pb kidney levels inherent to the metal mixture-treated group reflect additive or synergistic GSH depletion and attenuation of this transport modality, resulting in decreased As excretion.

3.5. Conclusions

Treatment of rats with a metal mixture of Pb, As and Mn modified Pb's deposition in several organs as compared to its deposition in rats treated with Pb alone. The most marked increase of Pb levels was observed in kidney and brain. In addition, in the metal mixture-treated rats, blood Pb levels fail to reflect increased Pb deposition in kidney and brain, raising concern that blood Pb levels may underestimate risk associated with Pb-induced kidney and brain damage. Selected endpoints of nephro- and neurotoxicity should be investigated to support the proposal of re-evaluation of Pb exposure limits in this metal mixture.

Chapter 4

Biomarkers as tools to investigate the toxic effects induced by a mixture of lead, arsenic and manganese in a rat model

4.1. Background

BMs, as observable endpoints in a continuum of events from exposure to disease, are becoming increasingly important tools for the detection and diagnosis of early toxicity (Kakkar and Jaffery, 2005; Scherer, 2005) as well as studies of the interactions among metals (Wang and Fowler, 2008). To date, the control of a mixture exposure has been mostly performed through the BMs of exposure and/or effect used in routine for each single metal of the mixture (ASTDR, 2000). Despite the existence of several BMs for Pb, As or Mn induced toxicity (Cowan et al, 2009; Heitland and Köster, 2003; Higashikawa et al, 2000), practically no information exists concerning the interactions of these three metals with the potential changes on animals or human neurotoxicity.

4.1.1. Biomarkers of exposure

Blood Pb concentration is the most used BM of exposure to Pb (ASTDR, 2007b; Batterman et al, 2011; Fukui et al, 1999; Patrick, 2006), although this parameter reflects mainly the history of the previous few months of exposure (Casarett and Doull's, 2007). Several attempts have been made to use urinary Pb as a surrogate of blood Pb levels, but this approach is of limited value since it is not applicable on an individual basis (Fukui et al, 1999). Since As is rapidly cleared from blood, measurements of blood As, reflect exposures only within a very recent past or to relatively high-levels of the metal (ASTDR, 2007a; Marchiset-Ferlay et al, 2012). Urinary As measurements are considered a reliable exposure BM, because urinary elimination is the major route for its excretion (de Vizcaya-Ruiza et al, 2009). Blood and urinary Mn levels can only indicate average levels of exposure on a group basis, not being suitable to be used individually (ASTDR, 2007c; Bader et al, 1999; Cowan, 2008). Moreover, urinary Mn levels are even less valuable BMs, since elimination of

Mn occurs primarily via biliary excretion to the feces (ASTDR, 2007c; Batterman et al, 2011; Santamaria, 2008).

4.1.2. Biomarkers of effect

The study of the mechanisms of metals neurotoxicity is the starting point to select BMs of neurotoxic effects (Costa, 1996); several authors have reported how the changes in different systems, such as the cholinergic, dopaminergic and even the hematopoietic system (Bhadauria and Flora, 2004; Maines, 1980; Montes et al, 2011; Patrick, 2006; Pohanka, 2014) can be involved in neurotoxicity.

Cholinesterases can be inhibited by heavy metals (Vidal-Liñán et al, 2014), existing however an elevated number of published dissimilar results pertaining to Pb, As or Mn effects in brain AChE activity. Pb can dose dependently inhibit AChE activity in brain cells and in vitro, when at high concentrations the metal seems to influence protein folding through a process involving direct contact with the enzyme (Richetti et al, 2011). Paradoxically, AChE activity in the brain of zebra fish is inhibited even at low Pb concentrations, being suggested that the effect of Pb could be mostly due to interference with brain metabolism/physiology leading to an indirect effect on AChE activity (de Lima et al, 2013). The exposure to As decreases the activity of AChE, but a significant augment in its brain activity was also found (Herrera et al, 2013). It is the proposal of some authors that ACh would accumulate in an initial phase, causing an increase in AChE, in order to degrade the excess of the neurotransmitter, but due to a prolonged effect of As on synaptic receptors, less ACh would be produced causing a progressive decrease in AChE (de Lima et al, 2013). Mn exposure may also produce differential effects on AChE, with experimental high sub-acute Mn doses inducing increased brain AChE activity, whereas a decreased activity of the enzyme was also observed in a long term chronic treatment (Michalke and Fernsebner, 2013). AChE is highly expressed in the CNS, is present in the skeletal muscle end plate and can also be

peripherally found in erythrocytes, where its function is not fully understood (de Lima et al, 2013, Worek et al, 1999). There has been a conviction that its measurement could serve as a peripheral surrogate dose–effect index of neurotoxicity on cholinergic function. However butyrylcholinesterase (BChE), another cholinesterase, is also present in tissues were AChE can be found, such as muscles, brain and also in plasma (Marek et al, 2011). Therefore, caution is advisable when interpreting results pertaining to whole blood determinations and methods ensuring the determination of AChE alone are definitely advisable.

The dopaminergic system is a major target for environmental neurotoxicants, such as metals and DA is known to regulate the release of the hormone PRL (Montes et al, 2011). Decreased DA receptor density in the pituitary was already found in rats upon exposure to Pb, consistent with the elevated serum PRL concentrations (Smith et al, 1998). Also As can affect dopaminergic functions (Rodríguez et al, 2010) but few information exist about the effects of As on PRL release, while elevated serum PRL upon Mn exposure have been mentioned as suggesting DA disturbance (Montes et al, 2011). Generally to date, the results pertaining to the effect of metals on human serum PRL are still confusing, since negative studies on the association of PRL levels with exposure to neurotoxicants are also published. Moreover, PRL can be affected differently by different chemicals and the same chemical may cause different effects on PRL, depending on the exposure levels (Alessio, 2006). Exposure to multiple metals was already associated with PRL changes (Meeker et al, 2009).

The interference with enzymes of heme biosynthesis, ALAS and ALAD, leads to increased delta-ALA in circulating blood and urine (Gurer and Ercal, 2000). The best-known hematological effect of Pb is the interference with ALAD (ATSDR, 2007c; Patrick, 2006), which is also highly sensitive to As (Bhadauria and Flora, 2004). However little is known about the potential of Mn to interfere with heme biosynthesis. Anyway, it is achieved that delta-ALA accumulates especially in the liver and in the

brain (Onuki et al, 2004) where may cause CNS damage and peripheral neuropathy (Ryter and Tyrrel, 2000).

When heme metabolism disorders occur, such as the induced by metals, characteristically porphyrin levels increase in blood and urine (Fleck et al, 2003; Solinas and Vajda, 2008; Quintanilla-Vega, et al, 1996). Excessive accumulation of these heme precursors in the body have been considered responsible for neuropathy and neuropsychiatric symptoms that occur in hereditary hepatic porphyrias, despite the exact pathogenic mechanism is not clear (Ennis et al, 2003; Simon and Herkes, 2011). Pb exposure typically results in protoporphyrin accumulation in the erythrocytes and increased urinary excretion of coproporphyrin (Quintanilla-Vega, et al, 1996). Changed excretion patterns of urinary porphyrins, uro- and coproporphyrins have also been observed in vivo upon exposure to As via drinking water (Krishnamohan et a, 2007; Wu et al, 2004) and also in exposed workers (Ng et al, 2005; Krishnamohan et al, 2007). Despite the lack of information respecting to the eventual effect of Mn in heme biosynthesis or changes induced in urinary porphyrins, a few reports mention altered levels in liver and brain (Maines, 1980). Because different metals can exert their effects at different points of the metabolic pathway of the heme biosynthesis, changes in the urinary porphyrin profile can be used to differentially diagnose some specific metal exposures, with authors suggesting that porphyrins can be promising BMs of toxicity induced by metal mixtures (Wang and Fowler, 2008).

Pb, As and Mn co-occur in specific occupational settings and the three elements have the shared feature of inducing neurotoxicity. Considering these informations the present study was designed to assess the changes on neurotoxic effects induced by the co-exposure to Pb, As and Mn, through the evaluation of neurobehavioral and neurochemical changes in rats single metals exposed and in rats co-exposed to the mixture. To achieve this goal, motor activity assays were performed and several brain and peripheral biomarkers were determined.

4.2. Experimental Procedure

4.2.1. Chemicals

Chemicals were obtained from the following sources: 2-Propanol for HPLC (C_3H_8O; 99.5%), 5,5 –dithiobis (2-nitrobenzoic acid) (DTNB), acetylthiocholine (ATCh), diethyl ether ($C_4H_{10}O$), ethanol (C_2H_6O), ethopropazine, ethyl acetate ($C_4H_8O_2$), ethyl acetoacetate p.a. ($C_6H_{10}O_3$), methanol HPLC grade (CH_4O; ≥99.9%), phosphoric acid (H_3PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4) and Triton X 100 from Sigma–Aldrich; perchloric acid ($HClO_4$), potassium dihydrogen phosphate (KH_2PO_4), delta-aminolevulinic acid standard, sodium acetate ($C_2H_3NaO_2$), and p-dimethylaminobenzaldehyde ($C_9H_{11}NO$) from Merck; acetic acid ($C_2H_4O_2$) and hydrochloric acid p.a. (HCl; 37%) from Panreac; Iodine resublimed from BDH; Pure standards of uro-, hepta-, hexa-, penta- copro- and protoporphyrins (10 nM) where obtained from Porphyrin products, Frontier Scientific.

4.2.2. In vivo assay

A repeated exposure in vivo assay (8 days) with Wistar rats was performed as described in Fig.3.1 (Chapter 3).

4.2.3. Behavioral assays

Motor activity was assessed before the beginning of the experiment and 24-h after the administration of the last dose, through open-field behavior evaluation (Marreilha dos Santos et al, 2011) (Fig.3.1, Chapter 3). The tests were carried out in an

open-field apparatus consisting of a white box with a surrounding 30-cm-high opaque wall, the floor measuring 60 cm × 90 cm and divided in equal squares by black lines. Immediately after placement in the center of the open-field, the rats' movements were scored for five minutes. Two behavioral parameters were determined: the number of squares crossed with all paws (ambulations) and the number of times that both forelegs were raised from the floor (rearings). The rats were tested individually, and after each session, the open field was thoroughly cleaned with humid cleaning tissue.

4.2.4. Pb, As and Mn levels in brain, blood and urine

Determinations were performed as previously described in Chapter 3.

4.2.5. Acetylcholinesterase activity in brain and blood

Brain and total blood AChE activities were determined according to an adaptation of Ellman's method (1961) by Worek et al (1999) (Naik et al, 2008). This method is based on the formation of a yellow 5-thio-2-nitrobenzoate anion produced in the reaction between 5,5 -dithiobis-(2-nitrobenzoic acid) (DTNB) and thiocholine, after AChE-mediated hydrolysis of ATCh. The procedure consisted in the addition of 0.9 mL of buffer sodium phosphate dibasic/sodium phosphate monobasic (0.1 M) pH 7.4 and 0.1 mL of 5% Triton to 50 mg of brain homogenates in a Potter-Elvehjem. The reaction was initiated after the addition of 0.05 mL of DTNB (0.33 mM) in the presence of 20 µM ethopropazine (an inhibitor of BChE) and 0.05 mL of ATCh (1.56 mM), which was used as substrate. The reaction was measured for 3 minutes by the increase of absorbance in intervals of 10 sec at 412 nm, using a Hitachi spectrophotometer. For blood determinations, readings were performed at 436 nm to reduce interference by hemoglobin. The activity of AChE was calculated according to

the following formula: AChE activity = (Δ average*0.0015)/ (0.00017* tissue weight), with Δ = absorbance t_n – absorbance t_{n-1} and t = time. A calibration plot with R2=0.96 was used and precision expressed as coefficient of variation (CV %) was 12.2%. The results are expressed as nmol AChE/min/g brain protein and nmol AChE/min/g blood.

4.2.6. Prolactin in serum

Serum PRL was quantified by an enzyme immunoassay (Citomed ®). Plates were read in an Anthos Zenyth 3100 microplate detector. The QL was 0.6 ng/mL.

4.2.7. Delta-aminolevulinic acid in brain and urine

One mL of supernatant (2500 rpm, 10') from each sample (urine or brain homogenates) was added to 1 ml of acetate buffer (pH 4.6) and 0.2 mL of acetoacetate. The samples were mixed (5'') and incubated (100^{o}C, 10') followed by the addition of 3 mL of ethyl acetate, agitation (15'') and centrifugation (2000 rpm, 3'). A colorimetric reaction was started with Ehrlich reagent and the obtained organic phase. Delta-ALA concentrations were determined at 553 nm with an Hitachi spectrophotometer. Calibration curves were generated daily with a delta-ALA standard. The QL was 0.012 mg delta-ALA/L and the results are expressed as mg of delta-ALA per g of urinary creatinine or per g of brain protein.

Urinary levels of creatinine were determined by a colorimetric method with a Randox (CR510) commercial kit, and brain protein contents according to the method described by Bradford (1976).

4.2.8. Total porphyrins in urine

70µL of acetic acid and 1750 µL of diethyl ether were added to 700µL of each urine sample, agitated (15'') and centrifuged (2500 rpm, 2'). After the centrifugation, 1750µL of an iodine chloridric solution (1% in HCl 5%) was added to the organic phase and after new agitation and centrifugation the aqueous phase was placed in a water bath (37^{o}C, 5'). Absorbances were measured at 380nm and 430nm, corresponding to impurities' maximum absorbances, and at 401nm, in a Hitachi spectrophotometer. Total porphyrins are expressed in µg/g creat and were calculated according to the expression proposed by Soulsby and Smith (1974): Porphyrins (µg/L) = [2 x A_{401} – (A_{430} + A_{380})] x 2,093 x 1,064 x 1000, and corrected for creat.

4.2.9. Brain and urinary porphyrin profile

For the analysis of brain and urinary porphyrin profile, 20 µL of HCL 10 M was added to 750 µL of each sample (urine or brain homogenates), agitated in the vortex and kept in the dark for 30'. After centrifugation (2500 rpm, 10') the supernatant was stored at -80^{o}C until analysis (Woods et al, 2004). Given that porphyrins are photosensitive compounds, during the handling of all the samples protection from light was assured.

Chromatographic porphyrin analysis was performed by High Performance Liquid Chromatography (HPLC) in a Hewlett Packard Agilent 1100 HPLC system equipped with a quaternary pump, solvent degasser, fluorescence detector and sampler (50 µL). Separation was accomplished on a LiChrospher 100 Merck RP18 column (125mm x 4mm, 5µm), fitted with an identically packed column (4 x 2 mm) maintained at ambient temperature. The mobile phase consisted of methanol 100% (solvent A) and sodium phosphate monobasic (50 mM), pH 3.5 (solvent B). A gradient was used starting at the mobile phase A:B (30:70%) until 3 min, changed linearly to A:B (80:20%) until 10 min, maintained equal for 3 more minutes and after, the column was

equilibrated with 30% of solvent A for 5 min. The flow rate was 1.0 mL/min. The fluorescence detector was set at an excitation wavelength of 395nm and an emission wavelength of 620nm. Data acquisition, processing and instrument control was performed by a Chemstation software.

A dried porphyrin standard (10 nM) was recovered with HCl (3M) and several dilutions of this stock solution were used to obtain a calibration plot with R^2=0.9998. The determined QLs were: 7.7 nmol/L for uroporphyrins, 10.2 nmol/L for heptaporphyrins, 11.0 nmol/L for hexaporphyrins, 10.6 nmol/L for pentaporphyrins, 17.2 nmol/L for coproporhpyrins and 10.9 nmol/L for protoporphyrins.

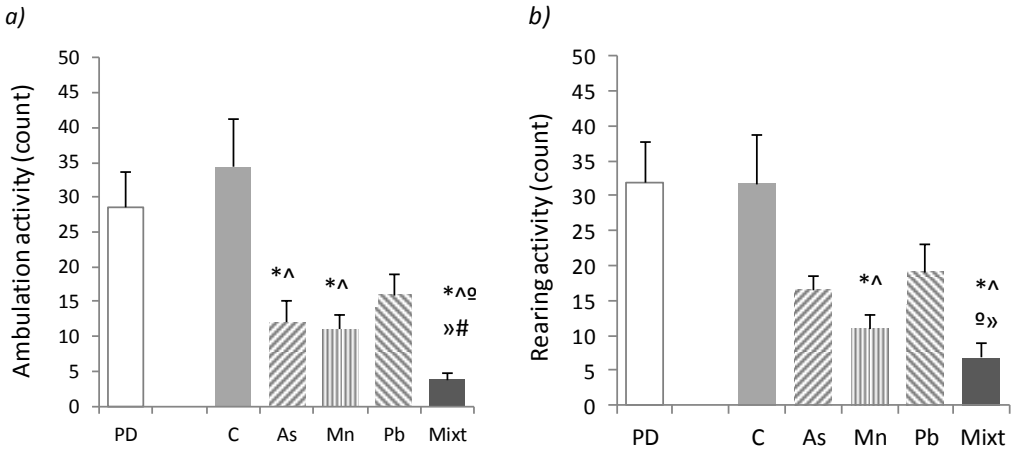
All the results are expressed as nmol of porphyrin per g of urinary creat or per g of brain protein.

4.2.10. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SPSS 16.0 statistical package for Windows (SPSS, Inc., Chicago, IL, USA). After verification of data adequacy for parametric methods, normal distribution by Kolmogorov-Smirnov test and homogeneity of variance with Levene's test, non-parametric analysis revealed to be a better statistical approach. All groups were compared by Mann-Whitney tests. The significance of the results was considered when p values were less than 0.05.

4.3. Results

4.3.1. Behavioral assays



Figs.4.1 (a) and (b): Pb, As and Mn and Pb/As/Mn mixture effects in motor activity, ambulation (a) and rearing (b) counts. Data represent the mean ± SD of ambulation and rearing counts before (PD) and after the treatments in C, As, Mn, Pb and Mixt groups. N=6 each group. All the groups were compared by Mann-Whitney U tests: *, ^, º, » and # are $p < 0.05$ versus PD, C, As, Mn and Pb.

Two parameters of motor activity were determined, ambulation and rearing, prior to treatments and 24 h after the last dose. In the Pb treated group a trend towards decreased motor activity was observed when compared with the control group and with the PD group (Figs.4.1a and b). The As treated group revealed similar trends to those found in the Pb treated group, but with statistical significance for ambulation ($p< 0.05$) (Fig.4.1a). The Mn treated rats exhibited a significant decrease in ambulation and rearing compared with the two control groups ($p < 0.05$) (Figs.4.1a and b). Rats treated with the metal mixture showed a decrease in both motor parameters that were significantly different from all the other

groups ($p < 0.05$) (Figs.4.1a and b), except for rearing activity when compared with the Pb treated group ($p < 0.05$) (Fig.4.1b).

4.3.2. Brain biomarkers

Acetylcholinesterase activity in brain

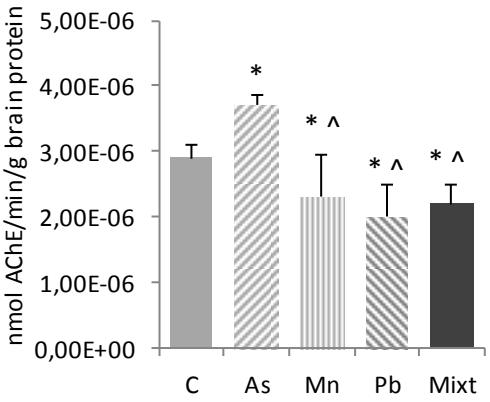


Fig.4.2: Brain AChE activity in Pb, As and Mn and Pb/As/Mn mixture treated groups. A control (C) group was also used. Data represent the mean ± SD and n=6 each group. All the groups were compared by Mann-Whitney U tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

Rats treated with Pb or Mn exhibited a significant ($p < 0.05$) decrease in the activity of the enzyme with respect to controls and the As group (Fig.4.2). Inversely the activity of AChE in the brain of the As single treated rats was significantly ($p< 0.05$) increased as compared with controls (Fig.4.2). Brain AChE activity of the group exposed to the mixture was significantly ($p< 0.05$) lower than controls and As treated rats (Fig. 4.2).

Brain delta-aminolevulinic acid levels

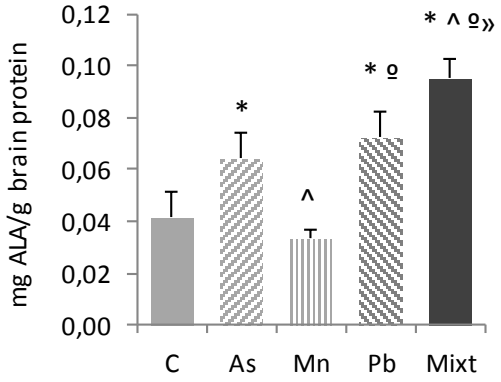
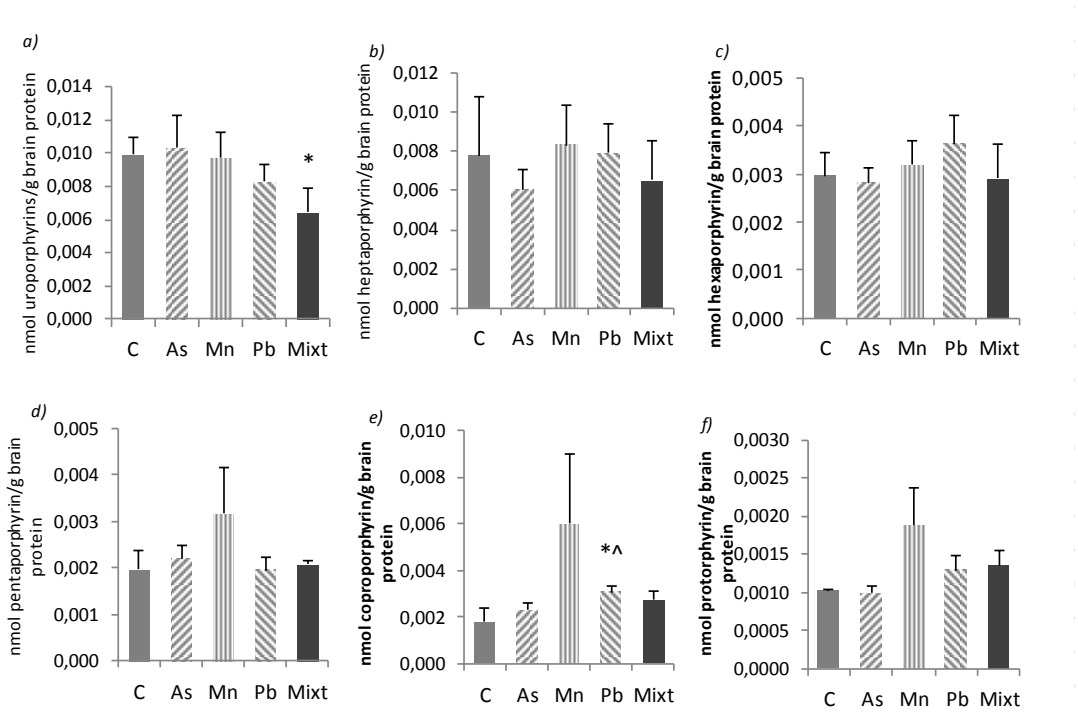


Fig.4.3: Brain concentrations of delta-ALA in Pb, As and Mn, and Pb/As/Mn mixture treated groups. A control (C) group was also used. Data represent the mean ± SD and n=6 each group. All the groups were compared by Mann-Whitney U tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

Brain delta-ALA concentrations were significantly increased in the Pb and As treated groups compared with the control ($p< 0.05$), whereas in the Mn treated group, delta-ALA brain levels were similar to controls ($p > 0.05$) (Fig.4.3). The highest concentrations of brain delta-ALA were observed in the metal mixture treated group, with levels significantly increased compared to all the other groups ($p< 0.05$) (Fig.4.3).

Porphyrins profile in brain



Figs. 4.4 (a), (b), (c), (d), (e) and (f): Uroporphyrin (a), heptaporphyrin (b), hexaporphyrin (c), pentaporphyrin (d), coproporphyrin (e) and protoporphyrin (f) levels in the brain of controls (C) and Pb, As, Mn and Pb/As/Mn mixture treated groups. N=6 each group. Data represent the mean ± SD. All the groups were compared by Mann-Whitney U tests: *, ^, º and » are $p < 0.05$ versus C, As, Mn and Pb.

The porphyrin profile was analyzed in the brain of controls and exposed animals. With respect to the Pb treated group, its comparison with the controls leaded to note a decrease in uroporphyrin concentrations (Fig.4.4a), along with augments in the levels of hexa-, copro- and protoporphyrins (Figs.4.4c, e and f). All these differences lacked statistical significance, except those concerning to the coproporphyrins concentrations, where a significant difference was noted relatively to the controls and the As treated group ($p<0.05$). The As exposed group had very slight and not significant ($p>0.05$) increases of penta- and coproporphyrins (Figs.4.4d and e), when compared with the controls. Increased levels of penta-, copro and protoporphyrins were observed in the brain of the Mn exposed group as compared with controls (Figs.4.4d, e

and f). These differences observed were not significant ($p>0.05$) probably due to the high SD values attained for the group exposed to this metal. In the mixture exposed group the levels of uroporphyrins were lower than all the other groups, although this difference was significant only when compared with the controls ($p<0.05$) (Fig.4.4a). A trend for a slight increase of copro and protoporphyrins of the mixture treated group was also observed as compared with the control group (Figs.4.4e and f).

4.3.3. Peripheral Biomarkers

Acetylcholinesterase activity in blood

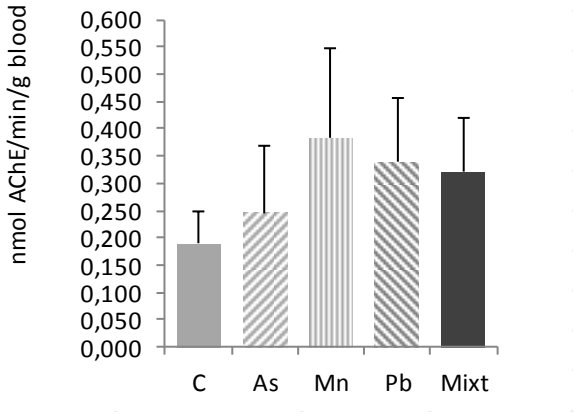


Fig.4.5: Total blood AChE activity in Pb, As and Mn and Pb/As/Mn mixture treated groups. A control (C) group was also used. Data represent the mean ± SD and n=6 each group. All the groups were compared by Mann-Whitney U tests.

No significant difference was found among groups concerning to AChE activity in the total blood (Fig. 4.5).

Serum prolactin levels

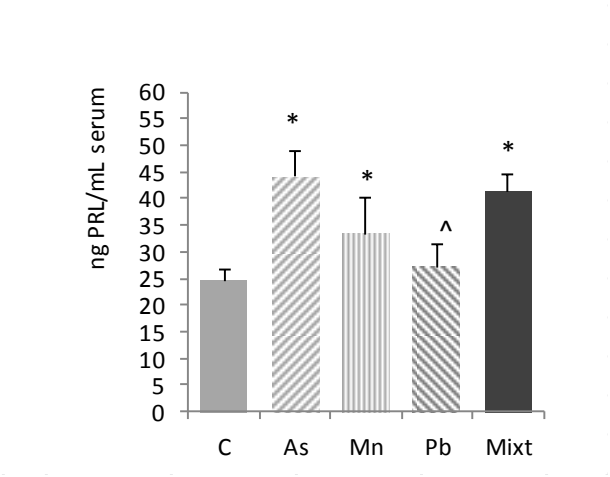


Fig.4.6: Serum PRL levels in Pb, As and Mn and Pb/As/Mn mixture treated groups. A control (C) group was also used. Data represent the mean ± SD and n=6 each group. All the groups were compared by Mann-Whitney U tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

In the Pb treated group the levels of serum PRL were significantly ($p<0.05$) lower than the As treated group (Fig. 4.6). Serum PRL levels were found to be significantly increased in As and in Mn single treated rats when compared with controls ($p< 0.05$) (Fig 4.6). The rats treated with the mixture exhibited an increase of serum PRL concentrations that was significantly ($p< 0.05$) different than the controls (Fig. 4.6).

Urinary delta-aminolevulinic acid levels

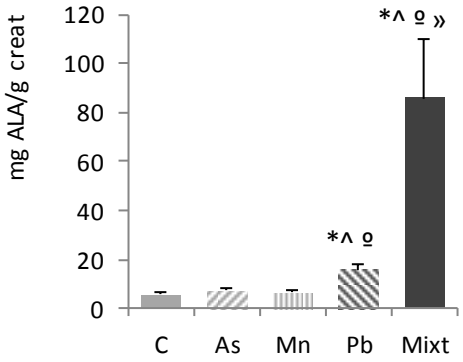


Fig.4.7: Urinary concentrations of delta-ALA in Pb, As and Mn and Pb/As/Mn mixture treated groups. A control (C) group was also used. Data represent the mean ± SD and n=6 each group. All the groups were compared by Mann-Whitney U tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

Delta-ALA-U levels were analyzed and corrected for creatinine content. The Pb and metal mixture treated rats showed a significant increase in urinary delta-ALA levels compared with all the other groups ($p < 0.05$) (Fig.4.7).

Urinary total porphyrins

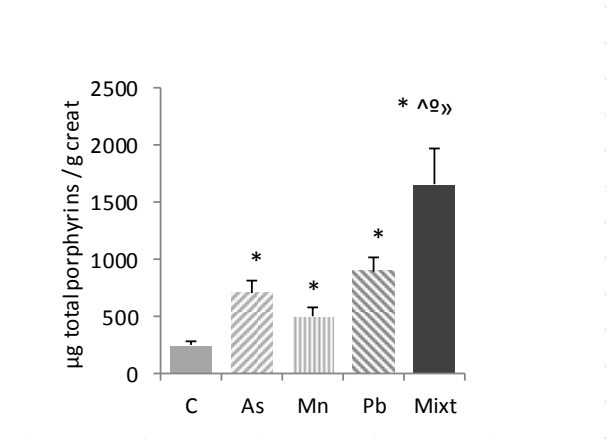
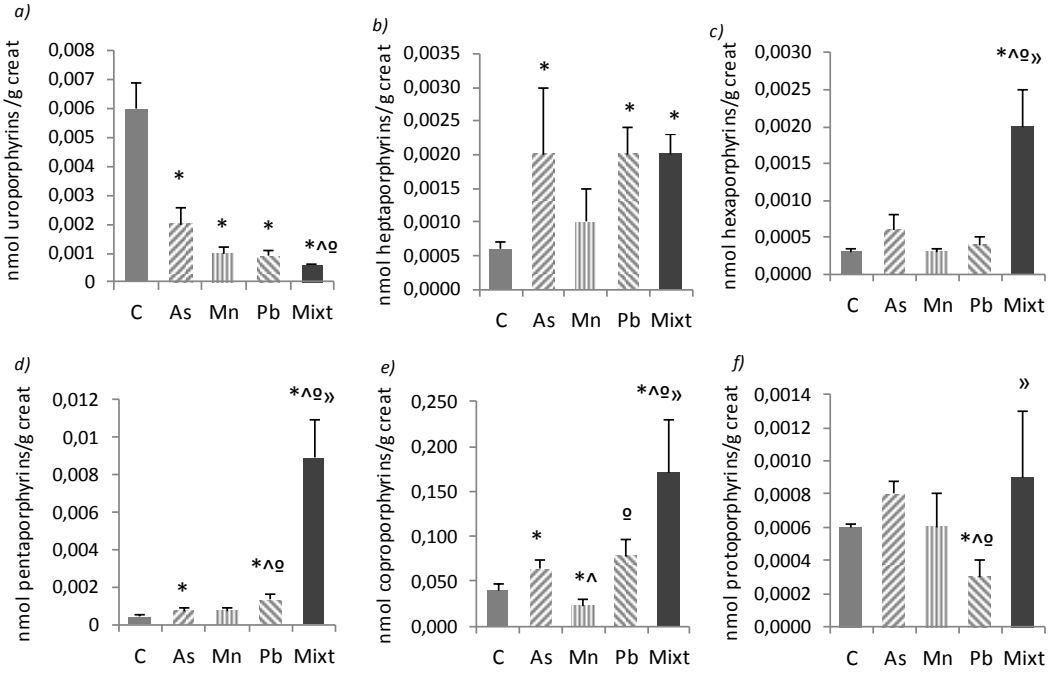


Fig.4.8: Urinary concentrations of total porphyrins in Pb, As and Mn and Pb/As/Mn mixture treated groups. A control (C) group was also used. Data represent the mean ± SD and n=6 each group. All the groups were compared by Mann-Whitney U tests: *, ^, º, and » are $p < 0.05$ versus C, As, Mn and Pb.

Total urinary porphyrins were determined, leading to the observation that all the single exposed rats had total urinary porphyrins significantly ($p<0.05$) higher than the controls (Fig. 4.8) and that the mixture treated group exhibited a significant ($p<0.05$) augment of this urinary parameter, when compared with the controls and all the single exposed groups (Fig. 4.8).

Urinary porphyrin profile



Figs.4.9 (a), (b), (c), (d), (e) and (f): Uroporphyrin (a), heptaporphyrin (b), hexaporphyrin (c), pentaporphyrin (d), coproporphyrin (e) and protoporphyrin (f) urinary levels of controls (C) and Pb, As, Mn and Pb/As/Mn mixture treated groups. N=6 each group. Data represent the mean ± SD. All the groups were compared by Mann-Whitney U tests: *, ^, º and » are $p < 0.05$ versus C, As, Mn and Pb.

The determination of the urinary porphyrin profile of the Pb treated group leaded to observe significant ($p<0.05$) decreases in the levels of uroporphyrins (Fig.4.9a) as compared with controls and in the levels of protoporphyrins when compared with controls and the other single exposed groups (Fig.4f). It was also noted that this group had significant ($p<0.05$) augments of heptaporphyrins as compared with controls (Fig.4.9b), of pentaporphyrins as compared with controls and the other single treated groups (Fig.4.9d) and also of coproporphyrins as compared with Mn exposed animals (Fig.4.9e). The rats treated with As showed significant ($p<0.05$) decreased levels of

uroporphyrins, when compared with the controls (Fig.4.9a) as well increased levels of all the other determined porphyrins, although the augments observed in hexa- and protoporphyrins were not statistically significant (Figs.4.9b, c, d, e and f). In comparison with the controls, significant ($p<0.05$) decreased levels of uro- and coproporphyrins were detected in the Mn treated rats (Figs.4.9a and e). Augments of hepta- and pentaporphyrins were also observed in the Mn group as compared with the control group (Fig.4.9b and d) despite lacking statistical significance ($p>0.05$). Concerning to the mixture exposed group, the levels of uroporphyrins were significantly ($p<0.05$) lower than all the other groups, with exception to the Pb treated group (not statistically significant) (Fig.4.9a). The urinary heptaporphyrins were significantly ($p<0.05$) higher than controls (Fig.4.9b); In addition, hexa-, penta- and coproporphyrins values were significantly ($p<0.05$) higher than all the other groups (Figs.4.10c, d and e). A trend toward an increase of protoporphyrins was also observed concerning to all the other groups, although this augment was only statistically significant ($p<0.05$) when compared with the group exposed to Pb (Fig.4.9f).

4.3.4. Biomarkers correlations

Biomarkers in brain

Table 4.1: Relationships between metal levels in brain (Br) and delta-ALA, AChE activity and uroporphyrin Br levels, in Pb, As, Mn and Pb/As/Mn mixture treated groups. Data represent significant correlations expressed by Spearman's rho values ($p<0.05$).

		Spearman's rho		
Group	Metals	ALA-Br	AChE-Br	Uro-Br
As	As-Br	-	-	-
Mn	Mn-Br	-	-	-
Pb	Pb-Br	-	-	-
Mixt	As-Br	0.627	-0.689	-0.743
	Mn-Br	0.811	-0.638	-0.735
	Pb-Br	0.783	-0.610	-0.837

Spearman's rho correlations were also determined between brain metal levels and delta-ALA, AChE activity and uroporphyrins in brain, leading to observe that no significant correlation was attained upon single exposure (Table 4.1). Inversely, in the mixture treated group, delta-ALA brain levels had a significant and positive correlations ($p<0.05$) with the three metals ($\rho=0.783$, $\rho=0.627$ and $\rho=0.811$, respectively pertaining to Pb, As and Mn brain levels) (Table 4.1). AChE activity in brain, showed a significant and negative correlation with Pb brain levels ($\rho=-0.610$; $p<0.05$), As brain levels ($\rho=-0.689$; $p<0.05$) and with Mn concentrations in brain ($\rho=-0.638$; $p<0.05$). Also, significant ($p<0.05$) negative correlations were determined between brain uroporphyrin levels and the concentrations of the three metals in this organ ($\rho=-0.837$, $\rho=-0.743$ and $\rho=-0.735$, respectively Pb, As and Mn levels) (Table 4.1).

Brain and peripheral biomarkers

Table 4.2: Relationships of peripheral BMs, urinary (U) As and delta-ALA levels, and Pb and Mn concentrations in blood (B), with their respective concentrations in brain (Br), in Pb, As, Mn and Pb/As/Mn mixture treated groups. Data represent significant correlations expressed by Spearman's rho values ($p<0.05$).

		Spearman's rho			
Group	Metals	As-U	Mn-B	Pb-B	ALA-U
As	As-Br	0.828	-	-	-
	ALA-Br	-	-	-	-
Mn	Mn-Br	-	0.741	-	-
	ALA-Br	.	-	-	-
Pb	Pb-Br	-	-	0.682	-
	ALA-Br	-	-	-	-
Mixt	As-Br	0.839	-	-	-
	Mn-Br	-	0.838	-	-
	Pb-Br	-	-	0.643	-
	ALA-Br	-	-	-	0.788

The single treated groups showed significant positive correlations between peripheral BMs and the concentrations of each respective metal in the brain, namely between Pb levels in blood and brain (ρ=0.682; $p<0.05$) in the Pb treated group, As levels in urine and brain (ρ=0.828; $p<0.05$) in the As treated group and Mn concentrations in blood and brain (ρ=0.741; $p<0.05$) in the Mn group (Table 4.2). The group exposed to mixture showed also positive and significant correlations between blood and brain Pb concentrations (ρ=0.643; $p<0.05$), urinary As and As levels in the brain (ρ=0.839; $p<0.05$), blood and brain Mn levels (ρ=0.838; $p<0.05$) and urinary and brain delta-ALA concentrations (ρ=0.788; $p<0.05$) (Table 4.2).

4.4. Discussion

Excessive exposure to metals can lead to brain dysfunction (Kordas et al, 2010) being established that Pb, As and Mn induce neurotoxic effects (Gerhardson et al, 1995; Health Canada, 2008, Rodriguez et al, 2003). Yet, despite the mixture of these elements is known to coexist in the environment, in particular in several occupational settings, the combined effects of metal mixtures is largely unknown (Tiffany-Castiglioni et al, 2006; Wang and Fowler, 2008). Herein, we evaluated the changes of neurotoxic effects in response to rats' treatment with each studied metal and with the mixture of Pb, As and Mn, through behavioral assays and several BMs were also determined. More specifically, rearing and ambulation counts were performed in motor activity assays and the following brain and peripheral biomarkers were determined: Pb, As and Mn levels in brain, blood and urine; brain and blood AChE activity; brain and urinary delta-ALA levels and porphyrin profile, urinary total porphyrins and serum PRL concentrations.

4.4.1. Motor activity

Open-field spontaneous motor activity assessment is a sensitive and reliable behavioral test; it has been previously used to assess Pb, As or Mn-induced neurotoxicity (Marreilha dos Santos, 2011; Moreira et al, 2001; Sansar et al, 2011). In this study, all single metal treated rats showed decreased motor activity compared with the controls, although not attaining statistical significance for either the motor parameters (rearing and ambulation) in the Pb treated group, neither for rearing in the As treated group (Figs.4.1 a and b). Conflicting results on ambulation and rearing activities in Pb-exposed rats have been previously noted (Chandra et al, 1981; Reddy et al, 2003; Rocha et al, 2001; Rodrigues et al., 1996), consistent with our inability to ascertain a clear and significant effect on behavioral changes in the Pb treated group. While reductions in these parameters have been previously reported as dose dependent (Chandra et al, 1981), the eventual existence of Pb threshold levels for a clear manifestation of behavioral effects possibly exists. With respect to As and Mn, our results corroborate previous reports, consistent with decreased motor activities (Rodríguez et al., 2010; Vezér et al., 2005), which were already associated with induced motor damages (Rodríguez et al, 2010; Vezér et al, 2005). Our data also show that the treatment with the 3-metal mixture decreases ambulation and rearing activities compared with all the other groups (Figs.4.1a and b). This is in agreement with earlier reports, where rats simultaneously co-treated with Pb and Mn showed a significant decrease in motor activity compared with single metal treated animals (Chandra et al., 1981). Motor deficits due to As exposure have also been described (Mejía et al., 1997). Combined, all data allows us to conclude that Pb, As and Mn mixture treatment exacerbates motor dysfunction in the rat.

4.4.2. Brain biomarker levels

Metals

It is well established that Pb, As and Mn perturb brain function (ATSDR, 2007a and c; Marreilha dos Santos et al, 2011). In the present study, Pb, As and Mn brain levels were significantly higher in each single treated group, as compared with the control (Fig. 3.4a, b and c, Chapter 3) corroborating early reports, where increased brain Pb, As or Mn levels upon exposure to these metals were associated with neurobehavioral deficits (Donoghue, 2004; Landrigan et al., 2006; Ramteke et al, 2009; Reckziegel et al, 2011; Rodríguez et al, 2003 and 2010). Notably, Pb brain levels in the metal mixture treated group were significantly higher than in any of the other groups (Fig.3.4a, Chapter 3). Consistent with our results, higher brain Pb levels were previously reported in rats exposed to the binary mixtures of Pb/Mn and Pb/As, compared to rats only exposed to Pb (Chandra et al 1981; Mejía et al, 1997). Combined, these observations suggest that co-exposure to Pb and other metals increase brain Pb accumulation. It has yet to be determined whether this effect is associated with upregulation of Pb-specific transporters, increased leakiness of the neurovasculature and/or reduced clearance of Pb in brain. In addition, As and Mn brain concentrations in rats treated with the metal mixture were higher than in controls (Fig. 3.4b and c , Chapter 3). These results suggest an association between brain co-accumulation of these three metals and decreased motor activity in rats co-exposed to this mixture.

Acetylcholinesterase activity

The rats exposed to Pb exhibited a significantly ($p<0.05$) decrease in brain AChE activity (Fig. 4.2), according to in vitro, in vivo and epidemiological studies, demonstrating the inhibitory effects of Pb on the enzyme (Ademuyiwa et al, 2007; de Lima et al, 2013; Finkelstein et al, 1998; Reddy et al, 2003) with whole brain AChE activity showing

marked depletions (Tripathi et al, 1997). Concerning to As, while decreased brain AChE activity occurred in rats exposed to 25 or 100 mg As/dL in drinking water for 4 months (Rodriguez, et al, 2003), sodium meta-arsenite (50 mg/L) via drinking water for 42 days to pregnant rats provoked a 20% increase in the activity of the enzyme. This difference was attributed to the dose and length of treatments (Herrera et al, 2013). In this work the As treated rats showed a significantly ($p<0.05$) increase of brain AChE activity (22%) as compared with controls (Fig. 4.2), possibly expressing a hormetic response upon exposure to an As low dose (Fig. 3.1, Chapter 3) (Herrera et al, 2013). Agreeing with mechanistic evidences of Mn induced toxic effects in cholinergic neurons (Finkelstein et al, 2007), such as decreased brain AChE activity (Santos et al, 2012), the activity of the enzyme significantly ($p<0.05$) decreased in Mn treated rats when compared with the controls (Fig.4.2). Yet, it is to mention that Mn exposure may produce differential effects on brain AChE activity with descriptions of its increased activity (21%) (Liapi et al, 2008). A complex figure seems to involve the relationships between Mn and cholinergic functions, where tight regulation mechanism to maintain the levels of this essential metal (Ascnher and Dorman, 2006) and genetics may be among modulating factors (Santos et al, 2012). No correlations between AChE activity in brain and the levels of metals in the organ were attained upon single exposures. Since Pb, As and Mn affect cholinergic functions (Ademuyiwa et al, 2007; Finkelstein et al, 2007; Roy et al, 2006) and brain AChE activity actually changed (Fig. 4.2), possibly the doses administrated were not enough to attain a clear relationship between cholinergic disarrays and metal accumulation in brain. Moreover, Mn has selectivity for dopaminergic neurons (Ellingsen et al, 2003) possibly in detriment to cholinergic neurons.

The mixture treated group decreased AChE brain activity values, with respect to controls ($p<0.05$), but this change was similar in Pb and Mn single treated rats (Fig 4.2). Interactions among the three metals in the brain resulted in increased Pb and Mn accumulation, with concomitant decreased As levels in this organ (Fig.3.4a, Chapter 3). The final effect of As-induced increased brain AChE activity, concomitantly with Pb- and Mn- inhibition of the enzyme can explain why AChE activity decreased without

additivity or synergism (Fig. 4.2). The significant correlations ($p<0.05$) of the three elements concentrations in the brain with the activity of AChE (Table 4.1) support this suggestion. Additionally, upon exposure to the mixture the combined effect of Pb and Mn on the cholinergic system was plausibly enough to contribute to the decrease of motor activity via AChE inhibition.

Delta- aminolevulinic acid

Pb interferes with heme synthesis, promoting ALAS and inhibiting ALAD activity, consequently increasing blood delta-ALA levels (Ennis et al., 2003) and As also decreases blood ALAD activity (Bhadauria and Flora, 2004). In agreement with the information that delta-ALA can accumulate in the CNS (Demasi et al, 1996), in the present study Pb and As treated rats showed increased levels of this compound in the brain, as compared with the controls (Fig.4.3). However, no correlations between brain delta-ALA and the levels of each of these metals in response to single metal treatment were noted (Table 4.1). It is likely that in Pb treated rats the administered doses were insufficiently high to reach the requisite brain levels that trigger significant delta-ALA accumulation. Consistent with this observation, the treatment failed to cause a significant behavioral dysfunction (Table 4.1 and Figs.3.2a and b, Chapter 3). Practically no information exists on the effects of Mn on delta-ALA brain levels and our results showed that Mn treated rats and controls have similar delta-ALA levels in brain (Fig.4.3), leading to think that at least for the administrated doses the metal does not have a relevant effect at this level.

In the metal mixture treated group, delta-ALA brain levels were the highest and significantly different ($p<0.05$) from all the other treatment groups (Fig.4.3). Moreover, brain delta-ALA concentrations were correlated with increased brain Pb, As and Mn levels (Table 4.1). Moreover brain Pb concentrations were higher in rats treated with a mixture of these metals than after single metal exposure, which suggests that Pb plays a key role in the excessive accumulation of brain delta-ALA. It is recognized

that the bioavailability of Pb in tissues may be affected by the presence of high affinity Pb-binding proteins (Rocha et al, 1995). A higher retention of Pb in the brain along with the presence of As, may possibly explain why brain delta-ALA concentrations increased in this group more than upon single exposures. Delta-ALA´s deleterious effects in the brain have been broadly described (Ennis et al., 2003) and common neurotoxic mechanisms are shared with Pb, As and Mn; these include interference with GABAergic systems and the induction of OS, conditions known to interfere with motor behavior (Adonaylo and Oteiza, 1999; Anderson et al, 2008; Demasi et al, 1996; Erikson et al, 2004; Flora et al, 2005; Jomova and Valko, 2011; Lelli et al, 2005; Milatovic et al, 2009; Reckziegel et al, 2011; ; Rodríguez et al, 2010; Struzynska and Sulkowski, 2004). Given the existence of shared mechanisms for delta-ALA, Pb, As and Mn induced neurotoxicity, the behavioral changes are likely multifactorial and stem from (1) the increase of Pb levels in brain and its deposition along with As and Mn, and (2) the accumulation of delta-ALA.

Porphyrin profile

Approximately 85% of heme is synthesized in the bone marrow and the remaining 15% is produced in the liver and other tissues, including the brain (Quintanilla-Vega et al, 1996; Maines, 1980); The presence of ALAS and other heme biosynthesis enzymes in this organ is previously reported, as well as its ability to carry out hemeprotein activities (Quintanilla-Vega et al, 1996); in our study, in all the groups, including controls, porphyrins were present in the brain(Fig 4.4). The group exposed to Pb had significantly ($p<0.05$) increased coproporphyrin levels in the brain as compared with the controls (Figs.4.4e and f), being known that coproporphyrinogen oxidase is inhibited by Pb (Ahamed and Siddiqui, 2007; Gurer and Ercal, 2000; Moore, 1998). No significant modifications were observed in As or Mn exposed groups despite data exists indicating that the presence of Mn in the brain can result in increased porphyrin levels (Maines, 1980). In addition, the mixture treated group exhibited a significant ($p<0.05$) decreased concentration of uroporphyrins as compared with the controls (Fig.

4.4a). The levels of brain porphyrins result from the balance of intricate processes, such as the control of their entrance from circulation and the regulation of its synthesis inside this organ, with several mechanisms ensuring the homeostasis of heme metabolism (Maines, 1980). Moreover, both neurons and glia synthesize different porphyrins at different rates and the degree and the extent of inhibition of porphyrins production can also be very different between these cells (Juknat, 1995). These informations lead to realize that the final outcome is a complex pattern of results, attained in this work by a trend for an accumulation of heme precursors in the brain of treated rats, although without very marked changes of individual porphyrins.

4.4.3. Peripheral biomarkers

The use of peripheral BMs, generally in blood and urine, is a common method to access modifications in inaccessible organs such as the brain, with the additional advantage that they represent simpler and cheaper methods to evaluate neurotoxicity in humans, surrogating the application of batteries of neurobehavioral tests. In this perspective, the levels of metals in blood and urine, urinary delta-ALA, total porphyrins and the porphyrin profile, blood AChE activity and serum PRL were determined.

Metals in blood and urine

Concerning to single exposed groups, positive and significant ($p<0.05$) correlations were determined: in Pb treated rats between Pb blood concentrations and Pb levels in brain; in the As treated group between urinary and brain As levels; and in the Mn treated group, Mn blood concentrations correlated with the levels of the metal in the brain (Table 4.2). Blood Pb concentration is actually one of the most widely used parameters for general clinical use and public health surveillance (ASTDR, 2007b) in detriment of urinary Pb, since excretion via urine is not the main route of Pb excretion

(Casarett and Doull's, 2007). Urinary elimination is the major route for excreting As and thus urinary As measurements have been considered more reliable than blood As levels and are the most used BM to assess exposure (ASTDR, 2007a; Marchiset-Ferlay et al, 2012). With respect to Mn, although the weak suitability of using its blood or urinary levels as BMs (Phoon, 1988), urinary Mn seems to be the weakest parameter, since urinary excretion of Mn represents no more than approximately 1% (Santamaria, 2008). In this work, for both single and co-treated groups, the levels of peripheral metal levels, blood Pb and Mn and urinary As, correlated significantly ($p<0.05$) and positively with the concentrations of each respective metal in the brain (Table 4.2), suggesting that these non-invasive BMs can be used to indirectly assess their levels in this organ.

Acetylcholinesterase activity in blood

With respect to the whole blood AChE activity no significant differences were attained between groups, due to the high standard deviation values (Fig 4.5). AChE is found mostly in the nervous tissue and is also present in the membrane of red blood cells (RBCs). There is considerable confusion in literature where authors often report that they had measured AChE in human plasma with ACh as substrate, where in fact they had measured also the activity of BChE, which is also present in the plasma (Pohanka, 2014; Worek et al, 1999). Therefore, when using whole blood samples it is crucial to assure that AChE alone is being measured. Methods using BChE specific inhibitors such as ethopropazine, which was used in this work, allows the determination of AChE activity in human whole blood (Naik et al, 2008). Also intriguing, is the fact that the same methodology was applied simultaneously to rats and humans' whole blood, leading to observe significant differences between exposed humans and controls (Chapter 6). There is also a great lack of published information regarding to the effect of these metals in blood AChE activity of rats and thus, further studies are needed to confirm these results. Even so, some questions can be raised such as if the results observed in humans were due to exposure to other metals.

Serum prolactin

Increased serum PRL in response to Pb exposure was previously related to depression of the inhibitory dopaminergic influence on pituitary PRL release in humans (Takser et al, 2004). However, the group treated with Pb did not show a significant modification of serum PRL concentrations (Fig. 4.6), which might be attributed to the used low doses. The levels of the hormone in serum increased significantly ($p<0.05$) in As treated rats, when compared with the controls. Little information exists respecting to the effects of As in PRL, except a recent report describing that 100 and 200 mg/L of sodium arsenite given to rats by drinking water for 28 days resulted in decreased plasma levels of PRL in a dose dependent manner (Jahan et al, 2012), which disagrees with our results. Possibly the use of plasma instead of serum could explain this difference, due to the presence of coagulating factors in the samples (Stuijver et al, 2012). Mechanistic evidences support the results obtained in this work, such as the As effects in the dopaminergic system where induced OS can promote DA auto-oxidation, the association of DA decrease with changes in motor activity upon exposure to As (Mejía et al, 1997) and the inverse relationship between DA and serum PRL levels (Marreilha dos Santos et al, 2011). Biochemical mechanisms underlying Mn toxicity include DA auto-oxidation (Smargiassi and Mutti, 1999) with elevated serum PRL upon exposure to the metal, suggestive of DA disturbances involving the hypothalamus–hypophysis axis (Montes et al, 2011). Accordingly, Mn treated rats had significantly ($p<0.05$) increased serum PRL levels relatively to controls (Fig 4.6).

The mixture treated rats exhibited an increase of PRL serum levels that was significantly ($p<0.05$) higher than controls, but was not different from the levels found in the groups exposed to each metal alone (Fig. 4.6). Pb was the metal present in the brain of mixture treated rats at higher concentrations (Fig. 3.4a, Chapter 3), and probably counteracted As and Mn effects on the hormone (Fig. 4.6). However, no correlations were observed between serum PRL and the levels of the three metals in

the brain. Further studies using brain BMs are necessary to elucidate in which degree this peripheral parameter can indicate metal levels brain status.

Urinary delta-aminolevulinic acid

Pb treated rats were the only single exposed group for which urinary delta-ALA levels changed significantly ($p<0.05$), augmenting with respect to controls and the other single exposed groups (Fig. 4.7). This modification was not correlated with brain delta-ALA concentrations (Table 4.2) possibly because of the low dose used in the experiment.

In a different way, the metal mixture treatment leaded to increased delta-ALA levels in urine, which were higher than the sum of the levels for each of the three single metals treated groups (Fig. 4.7), suggesting that the mixture treatment leads to delta-ALA-U changes that exceed the additive effects of the individual metals. In fact, along with the known effects of Pb on the accumulation of delta-ALA, its increased urinary excretion is described in As exposed subjects (Marchiset-Ferlay et al, 2012). Plausibly in the co-exposed rats, a decrease in heme levels induced delta-ALA accumulation through negative feedback control. These events might not be enough to significantly modify delta-ALA urinary levels of single exposed As rats, but may explain the results observed for the mixture exposed group. Additionally, the observed increased delta-ALA excretion in urine may also be supported by the information that delta-ALA reabsorption is a saturable process and takes place only under low delta-ALA concentrations (O'Flaherty et al, 1981). In this case, in the mixture group, and in accordance with our results, we believe that the reabsorption is minimal as the secretion pathway is dominating. In addition urinary delta-ALA levels correlated significantly ($p<0.05$) with brain delta-ALA levels (Table 4.2) suggesting the suitability of this peripheral BM to be used to assess delta-ALA in brain. In vitro experiments established that brain delta-ALA is slowly metabolized once incorporated

into neurons (Juknat et al, 1995). Therefore, we posit that blood-borne delta-ALA may enter in the brain, leading to its accumulation in the co-treated group.

Urinary porphyrins

Disorders of heme metabolism, characterized by accumulation of porphyrins and their precursors, can be induced by exposure to certain chemicals. Thus, alterations in the heme biosynthetic pathway are often used as an index of exposure and preclinical damage to a variety of toxic agents (Quintanilla-Vega et al, 1996). Therefore, total porphyrins were determined with the purpose of preliminary evaluate if the 3 metals could interfere with heme biosynthesis and if in co-treated rats the magnitude of changes were enough to further investigate porphyrin profiles. All single exposed groups had significant ($p<0.05$) increased excretion of these heme precursors when compared with the controls (Fig.4.8), which can indicate increased total porphyrins body burden. These results confirm that Pb and As interfere with heme synthesis (Krishnamohan et al, 2007, ASTDR, 2007b) and show for the first time that Mn induce modifications in the excretion of porphyrins. Additionally, the co-exposure to the three metals resulted in increased total porphyrin urinary levels, which were significantly ($p<0.05$) augmented with respect to controls and were also significantly ($p<0.05$) higher than after exposures to each element alone (Fig.4.8). These results suggest the convenience of studying the profile of heme precursors in the urine.

Urinary porphyrins profile

Pb is one of the most studied metals with regard to its toxic effects on the heme biosynthesis pathway. The group exposed to Pb exhibited changes in the urinary porphyrin profile, such as a significantly ($p<0.05$) decrease of uroporphyrins (Fig.4.9a) as compared with controls, possibly due to delta-ALA accumulation resulting from ALAD inhibition (Fig.4.9e) (Quintanilla-Vega et al, 1996). The metal can also

impair markedly coproporphyrinogen oxidase leading to increased urinary excretion of coproporphyrin (Gurer and Ercal, 2000), which increased significantly ($p<0.05$) in the Pb treated group as compared with Mn treated animals (Quintanilla-Vega, et al, 1996). Since Pb interference with FECH may provoke protoporphyrin accumulation in erythrocytes (Quintanilla-Vega, et al, 1996), the significant ($p<0.05$) decrease of urinary protoporphyrins in Pb exposed rats (Fig. 4.9f) demands further explanation. Previous works indicate that urinary porphyrin concentrations has the potential for use as an early warning indicator for chronic As exposure (Ng et al, 2005; Quintanilla-Vega et al, 2006), with coproporphyrinuria described in smelter workers (Krishnamohan et al, 2007) as well as significant modifications in uroporphyrin and coproporphyrin concentrations in urine in other human populations (Ng et al, 2005), although with discrepancies pertaining to the direction of these changes (Wu et al, 2004). An altered urinary porphyrin profile was also observed in the As treated group, where uroporphyrins decreased significantly ($p<0.05$) when compared with the controls (Fig.4.9a), along with a significant ($p<0.05$) increase of penta- and coproporphyrin excretion (Figs.4.9d and e), which may represents augmented body burden of these heme precursors. Barely no information exists on the effects of Mn in the heme biosynthetic pathway, except a reference mentioning that in vitro the metal may inhibit FECH (Hift et al, 2011) and in vivo, ALAS in the brain(Maines, 1980). Urinary uro- and coproporphyrins decreased, as compared with the controls ($p<0.05$) (Figs.4.9a and e) and thus, this work is one the few studies developed on this issue, suggesting that Mn can interfere with the synthesis of heme and enhances the need of further investigations. The most marked urinary porphyrin profile modifications were observed in the mixture exposed rats. In this group, uroporphyrin levels were significantly ($p<0.05$) lower than the other groups, despite lacking statistical significance ($p>0.05$) when compared with Pb treated rats (Fig.4.9a) and hexa, penta and coproporphyrins were significantly ($p<0.05$) higher than all the other groups (Figs.4.9c, d and e), suggesting that combined effects of Pb, As and Mn may result in higher heme synthesis disorders.

No significant correlations were obtained between brain and urinary levels of each porphyrin since despite a trend for an accumulation of heme precursors in the brain of exposed rats not very marked changes of individual porphyrins were attained. Circulating heme precursors can enter the brain being known that delta-ALA and PBG, mainly accumulated in the liver, can cross the BBB (Andrade et al, 2013). In vitro studies show also that exogenous delta-ALA can be taken up by neuronal cells and be metabolized to PBG and porphyrins (Juknat et al, 1995) and indeed, the group treated with the mixture exhibited an accumulation of delta-ALA in the brain (Fig. 4.3). However, a tight regulation of brain porphyrin levels exists as well. The brain is normally fairly well protected from changes in plasma delta-ALA concentration by the very low BBB permeability to this compound and by a saturable efflux mechanism present at the choroid plexus (Ennis, 2003). Thus, the final outcome is very acceptably a complex pattern of results and may explain why individual porphyrins urinary levels poorly reflect changes in brain porphyrins.

4.5. Conclusions

This work established that the co-exposure to As, Mn and Pb induce increased behavioral toxicity and brought also insights of mechanistic interest, concerning to consequences of Pb, As and Mn interactions in vivo on neurotransmitter and heme precursors levels. Indeed the co-exposure to these elements induces modifications in the brain such as decreased activity of AChE and increased accumulation of delta-ALA, which can act as a neurotoxin and accumulate more than upon single exposures. In addition, changes in blood and urinary metal levels, increased serum PRL and urinary delta-ALA and total porphryrins were found. The obtained results are suggestive of the suitability of these parameters to be used as peripheral BMs of exposure and/or neurotoxicity upon exposure to the mixture of Pb, As and Mn, which will be investigated in the next chapter.

Chapter 5

Multibiomarker approach to identify the exposure to chemicals and predict the severity of neurotoxic effects

5.1. Background

Human biomonitoring has been increasingly recognized as a pragmatic methodology to control exposure to environmental/occupational pollutants (Pino et al, 2012). Neurotoxic effects induced by metals can be devastating on mental and physical functioning (Cannon and Greenamyre, 2011) leading to an urgent need to improve prevention, which assuredly involves the correct identification of the chemicals to which each person is exposed and/or the detection of earlier disease events. Yet, when metal concentrations in blood or urine are determined, these values do not give any information about the neurotoxic outcomes of each subject (Feron et al, 1995; Kordas et al, 2010). Therefore, great attention has been given to BMs of effect which can reflect physiological and morphological changes in cell and tissues resulting from the exposure and can be applied in exposed populations (Costa and Manzo, 1995). BMs are particularly useful in the evaluation of nervous system progressive diseases that manifest their symptoms long after the exposure to the initiating factors (Kakkar and Jaffery, 2005). In addition, due to the large variation in susceptibility to chemicals exposure, some individuals may experience toxic effects at levels that others can sustain without any problem (Bergdahl et al, 1997). Hence the evaluation of the magnitude of the neurotoxic effects in each subject is also vital. Paradoxically, the research of the neurotoxicity theme seems to be more slow than other fields with regard to biological monitoring and the development of markers of exposure and of health effects. The complexity of the nervous system and its peculiarities, as well as the fact that CNS disorders can arise from the dynamic disarray of several gene regulatory networks, proteins and metabolic alterations reflecting huge complex perturbations, are responsible for this limited advancement (Costa and Manzo, 1995). Besides, when pertaining to exposures to mixtures of metals with the inherent multi-mechanisms of metal toxicity, the knowledge about interactions of metal mixtures and BM endpoints are highly lacking (Wang and Fowler, 2008).

The growing need for a rigorous evaluation of new BMs is leading to the recognition that the use of appropriate statistical techniques is essential for the accurate evaluation of their clinical relevance. Several methodologies are used to select the most adequate BMs of exposure and/or effect on the determination of health risk in exposed populations. Correlation analysis is a common method, where it is determined whether a significant correlation exists between the levels of a biological parameter or endpoint(s) of disease and the exposure to specific chemical(s) (Casarett and Doull´s, 2007). In addition, an accurate BM must have the capacity to adequately identify or differentiate one condition (or outcome) from another. The diagnostic accuracy of a BM is most commonly measured by the calculation of its sensitivity and specificity. Sensitivity is the proportion of patients who are correctly categorized as exposed or having disease, among those who truly are exposed or have the disease; specificity is the proportion of patients who are correctly categorized as non-exposed or not having the disease, among all subjects who truly don't have the exposure or disease (SØreide, 2009). In this context, receiver-operating characteristic (ROC) curve analysis is emerging as a useful statistical tool to assess BMs' accuracy (Cai and Pepe, 2002; Doecke et al, 2012; Shin et al, 2013; SØreide, 2009) and has been previously applied for BMs of exposure to Pb (Sakai, 2000).

Concomitantly, some authors consider a naïve expectation that a single BM can capture the intricate process underlying a CNS illness (Quinones and Daouk, 2009) most particularly, when the illness is induced by more than one chemical, including mixtures of metals. Rather, exposed populations should be studied using a combination of exposure and effect BMs, to efficiently detect and diagnose early metal poisoning (Kakkar and Jaffery, 2005; Zhai et al, 2005). Lately the integration of BMs promise an improved diagnostic performance over single markers that may be lacking in sensitivity and/or specificity; this novel approach is being tempted in the early detection of CNS diseases through the use of multivariate statistical techniques (Ravid, 2009) such as discriminant and multiregression analysis (Da et al, 2014; List et al, 2013; Quinones and Daouk, 2009). Nevertheless, despite the promised improvements, analyzing several BMs can be laborious spending time and money, due to the need of

collecting more than one biological sample (e.g. blood and urine) and the use of more than one analytical technique. In this view, porphyrins constitute a group of distinct compounds (Fig. 1.1) intermediates of heme biosynthesis, which can be integrated as a multiparameter BM. The determination of a porphyrin urinary profile simply requires the collection of one urine sample (a noninvasive method) and one single HPLC analysis. In fact, Pb, As and Mn can induce porphyrins accumulation, that results from the interference with specific points of the heme metabolic pathway and when in excess, heme precursors are neurotoxic. These informations are suggestive that the integration of the several porphyrins might reflect global neurotoxic outcomes resulting from the action of each component of the mixture, possibly representing a promising alternative in the context of BMs integration.

The aim of this work was to generate predictive models to select and subsequently combine peripheral BMs. These BMs would first identify the type of exposure and after, predict the magnitude of neurotoxic effects induced by the mixture of Pb, As and Mn in an individual basis. The integration of peripheral BMs of exposure and/or effect is hypothesized to improve the prediction as compared with the traditional methodology based on the scrutiny of single BMs.

5.2. Methodology

Two procedures were created, the first one integrating the levels of the 3 metals in blood and/or urine, urinary delta-ALA and total porphyrins, blood AChE activity and serum PRL (procedure I) and the second one using the urinary porphyrin profile (procedure II). In each procedure the BMs were combined through statistical tools to create a 2 phase's prediction model, with phase 1 aiming to identify the type of exposure of each rat and phase 2 aiming to predict the severity of motor impairment.

To generate procedure I, the BMs were previously selected as described in the next section.

5.2.1. Selection of biomarkers

Data obtained from the in vivo assay with rats exposed to Pb, As, Mn or the mixture of the three metals were used to evaluate which BMs could better:

1) Distinguish Pb, As, Mn or Pb+As+Mn treated rats from controls;
2) Correlate with behavioral outcomes.

The BMs selected in 1) were further integrated as BMs of exposure and the BMs selected in 2) were combined as BMs of effect.

The evaluation of each BM's capability to distinguish exposed rats from controls was performed by the estimation of areas under ROC curves. The ROC curve is a plot of sensitivity, indicating the number of true positives (on the vertical axis) and specificity, indicating the number of true negatives (on the horizontal axis) for all possible thresholds of a BM in the study data set (Akobeng et al, 2007; Shin et al, 2009). Whereas a BM´s area value close to 1 indicates an excellent BM, a curve that lies close to the diagonal (Area = 0.5) has no information content and therefore no diagnostic utility. More than one ROC curve can be present in the same plot and thus the absolute areas under each curve were compared to determine which BM had the better diagnostic performance (Warnock and Peck, 2010). Correlation analysis was performed by the determination of Spearman´s rho coefficients.

5.2.2. Integration of BMs

Phase 1

Discriminant analysis was performed to build the phase 1 of each procedure (I and II). This statistical tool allows: the identification of variables that better discriminate two or more different groups of individuals; the use of these variables to create a discriminant function that represents in parsimonious manner the differences among the groups; and the use of discriminant functions to classify new individuals in the groups (Maroco, 2010). In fact discriminant analysis undertakes the same task as multiple linear regression by predicting an outcome using weighted combinations of X values (Muhameed and Saleh, 2014) (in this case the several BMs), but the outcomes are predicted for categorical variables (in this case the type of exposure, to Pb, As, Mn or its mixture). This is achieved through the generation of classification functions where different function coefficients of each BM are obtained for each group of rats. After applying all the functions to a subject under analysis, the group with the higher value of the classification function corresponds, according to the model, to the group where the subject belongs (Maroco, 2010). Discriminant analysis is known to be very robust to data assumption violations, such as the lack of normality, homogeneity of variances among groups and the absence of multicollinearity, but only if two conditions are guaranteed: the dimension of the smallest group must be smaller than the number of predictor variables and for each variable the means in each group cannot be proportional to its variances (Maroco, 2010). After verifying that these two conditions were assured, several combinations of the selected BMs were tested, achieving a compromise between the use of a minimum number of BMs and the maximization of predictive performance of the model, which was evaluated through the examination of canonical discriminant function graphics and classification results tables. The best solution was accomplished when the following BMs were used: in procedure I, urinary Pb, As and delta-ALA and the levels of Pb and Mn in blood; in procedure II all urinary porphyrins, uro-, hepta-, hexa-, penta-, copro- and

protoporphyrins. After this phase it was possible to identify if each rat was not exposed, single exposed to Pb, As or Mn or exposed to the mixture of the three metals.

Phase 2

The second phase of each procedure intended to evaluate the severity of the motor impairments of each rat, through the prediction of the number of ambulations and rearings. Multiple linear regression was performed, modeling the single response variable Y (ambulation or rearing counts) as a linear combination of the X variables (Helma, 2005) (the BMs). Neper logarithmic (ln) transformation was applied in all the X variables to approach them to regression model assumptions. Again, several combinations of selected BMs were tested, achieving the following compromise: absence of multicollinearity among the X variables, verified by the examination of Variance Inflation Factors (VIF) and tolerance values tables; minimization of the number of selected BMs; and maximization of the predictive performance of the model, evaluated through the examination of significance values ($p<0.05$) in ANOVA tables and R^2 values (Maroco, 2010). In procedure II, the levels of porphyrins in the brain were also integrated and evaluated with respect to their capability to predict motor activity in order to confirm the suitability of using the urinary porphyrins with the same purpose.

The same analysis was performed using each BM alone, for comparison with the multiparameter approach (appendix 1 and 2).

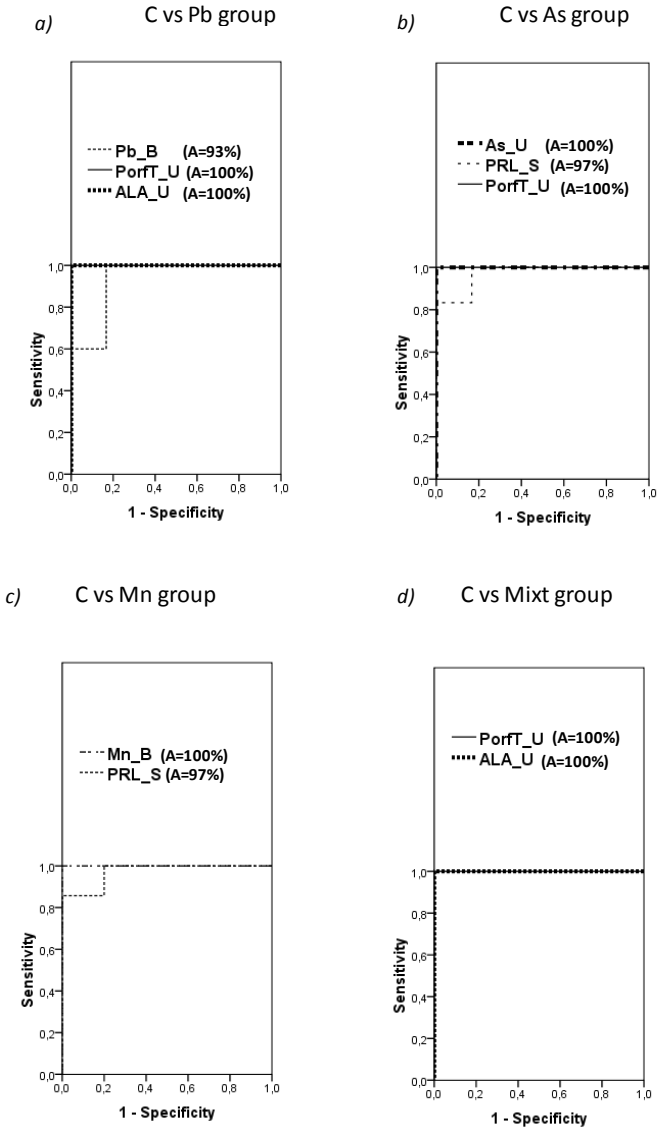
For all the statistics the significance of the results was considered when p values were less than 0.05.

5.3. Results

5.3.1. Selection of biomarkers

Phase 1

Biomarkers discrimination between exposed rats and controls



Figs.5.1 (a), (b), (c) and (d): The ability of peripheral BMs, Pb and Mn levels in blood (B), urinary (U) As, serum (S) PRL and urinary (U) total porphyrins (PorfT) and delta-ALA to discriminate between control rats (C) and the Pb treated group (a), C and the As treated group (b), C and the Mn treated group (c) and C and the mixture Pb/As/Mn treated group (Mixt). Curve's area (A) under the 1-Specificity (x) vs Sensitivity (y) axis are plotted by ROC (Receiver Operating Characteristics) analysis: areas are directly proportional to the BMs discriminant capabilities. A=100% indicates that all subjects were true positives and true negatives; N=6 each group. All the presented areas are significant ($p<0.05$).

Areas under the ROC curves were determined for all the peripheral BMs to evaluate their ability to differentiate controls from Pb, As, Mn or Pb/As/Mn mixture treated rats. In the Pb treated group significant area values ($p<0.05$) were noted for urinary delta-ALA (A=100%), total porphyrin levels (A=100%) and also for blood Pb levels (A=93%), showing the ability of these BMs to discriminate controls from Pb treated rats (Fig.5.1a). Urinary As or total porphyrin levels significantly ($p<0.05$) distinguished controls from As treated rats (A=100% for both BMs) as well as serum PRL (A=97%) (Fig.5.1b). Mn treated rats were significantly ($p<0.05$) differentiated from controls through the levels of Mn in blood (A=100%) or serum PRL ($p<0.05$) (A=97%) (Fig.5.1c). Urinary delta-ALA or total porphyrins discriminated between controls and mixture treated rats with statistical significance ($p<0.05$) both exhibiting areas of 100% (Fig.5.1d). For all the exposed groups no significant areas were observed when using urinary Pb or Mn levels, or As concentrations or AChE activity in blood (data not shown).

Phase 2

Correlations between brain biomarkers and motor activity

Table 5.1: Relationships between Pb, As, Mn, delta-ALA and AChE activity in the brain (Br), and motor activity, ambulation and rearing counts, in Pb, As, Mn and Pb/As/Mn mixture treated groups. Data represent significant correlations expressed by Spearman's rho values ($p<0.05$).

		Spearman's rho				
Group	Motor activity	As-Br	Mn-Br	Pb-Br	ALA-Br	AChE-Br
As	Ambulation counts	-0.737	-	-	-0.728	-
Mn		-	-0.555	-	-	-
Pb		-	-	-0.601	-	-
Mixt		-0.816	-0.811	-0.797	-0.783	0.745
As	Rearing counts	-0.614	-	-	-0.713	-
Mn		-	-0.623	-	-	-
Pb		-	-	-0.664	-	-
Mixt		-0.829	-0.847	-0.868	-0.791	0.709

Significant correlations between brain BMs and motor activity parameters (ambulation and rearing) were determined. In the Pb treated group, brain Pb concentrations were inversely correlated with both motor activity parameters (respectively ρ=-0.601 and ρ=-0.664 for ambulation and rearing counts; $p<0.05$) (Table 5.1). Negative Spearman´s coefficients were also determined in the As treated group, between As and delta-ALA brain levels and motor activity (respectively ρ=-0.737 and ρ=-0.728 for ambulation counts and ρ=-0.614 and ρ=-0.713 for rearing counts; $p<0.05$) (Table 5.1). In Mn treated rats, brain Mn levels correlated inversely with motor activity (ρ=-0.555 and ρ=-0.623 for ambulation and rearing counts; $p<0.05$) (Table 5.1). Additionally, in the mixture treated group, brain Pb, As and Mn levels correlated significantly with ambulation counts (ρ=-0.797, ρ=-0.816 and ρ=-0.811; $p<0.05$) and with rearing counts (ρ=-0.868, ρ=-0.829 and ρ=-0.847; $p<0.05$) (Table 5.1) as well as delta-ALA and AChE activity in brain (respectively ρ=-0.783 and ρ=-0.745, for ambulation counts and ρ=-0.791 and ρ=-0.709 for rearing counts; $p<0.05$) (Table 5.1).

Correlations between peripheral biomarkers and motor activity

Table 5.2: Relationships between peripheral BMs, urinary (U) As, delta-ALA and total porphyrins (PorfT) concentrations, serum (S) PRL and Pb and Mn levels in blood, and motor activity, ambulation and rearing counts, in Pb, As, Mn and Pb/As/Mn mixture treated groups. Data represent significant correlations expressed by Spearman's rho values ($p<0.05$).

		Spearman's rho					
Group	Motor activity	As-U	Mn-B	Pb-B	ALA-U	PRL-S	PorfT-U
As	Ambulation counts	-0.816	-	-	-	-0.799	-0.757
Mn		-	-	-	-	-0.610	-
Pb		-	-	-	-0.626	-	-0.674
Mixt		-0.893	-	-	-0.827	-	-0.909
As	Rearing counts	-0.698	-	-	-	-0.677	-0.697
Mn		-	-0.663	-	-	-0.636	-
Pb		-	-	-	-	-	-
Mixt		-0.833	-0.670	-	-0.780	-	-0.896

Several significant correlations were determined between peripheral BMs and motor activity parameters. The Pb exposed group exhibited negative associations of urinary delta-ALA and total porphyrin levels with ambulation counts (respectively ρ=-0.626 and ρ=-0.674; $p<0.05$) (Table 5.2). Respecting to As treated rats, urinary As, serum PRL and total urinary porphyrins correlated inversely with ambulation counts (respectively ρ=-0.816, ρ=-0.799 and ρ=-0.757; $p<0.05$) and with rearing counts (respectively ρ=-0.698, ρ=-0.677 and ρ=-0.697; $p<0.05$) (Table 5.2). In the Mn treated group correlations were determined between serum PRL and ambulation counts (ρ=-0.610; $p<0.05$) as well as Mn levels in blood and serum PRL correlations with rearing counts (respectively ρ=-0.663 and ρ=-0.636; $p<0.05$) (Table 5.2). The mixture treated group exhibited significant correlations of urinary As, delta-ALA and total porphyrin levels with ambulation counts (respectively ρ=-0.893, ρ=-0.827 and ρ=-0.909; $p<0.05$) and also of urinary As, delta-ALA, total porphyrin levels and Mn concentrations in blood with rearing counts (respectively ρ=-0.833, ρ=-0.780, ρ=-0.896 and ρ=-0.670; $p<0.05$) (Table 5.2).

5.3.2. Integration of biomarkers

Fig. 5.2: Procedure I proposed an individual assessment of the type of exposure and evaluation of motor activity deficits. Phase 1 allows to identify the type of exposure of each rat (to Pb, As, Mn or the mixture of these metals) through the determination of Pb and Mn in blood (B) and urinary (U) Pb, As and delta-ALA levels. Five classification functions (CF) were generated: for non exposed rats [CF (C)] and for exposures to Pb [CF (Pb)], to As [CF (As)], to Mn [CF (Mn)] or to the mixture of the three metals [CF (Mixt)]. The CF with the highest value indicates the type of exposure predicted by the model. Phase 2 evaluates motor impairment through the estimation of motor activity, ambulation and rearing counts. Depending on the type of exposure, after determination of total porphyrins in urine (Prof T U), serum prolactin (PRL S), Pb in blood (B) and/or AChE B activity, ambulation and rearing counts of each rat are predicted. R^2 coefficients and significance (p) values of each regression are also represented. NS means “Not significant”.

Individual assessment of exposure and severity of neurotoxic effects induced by Pb, As, Mn or the mixture of Pb/As/Mn
(Procedure II)

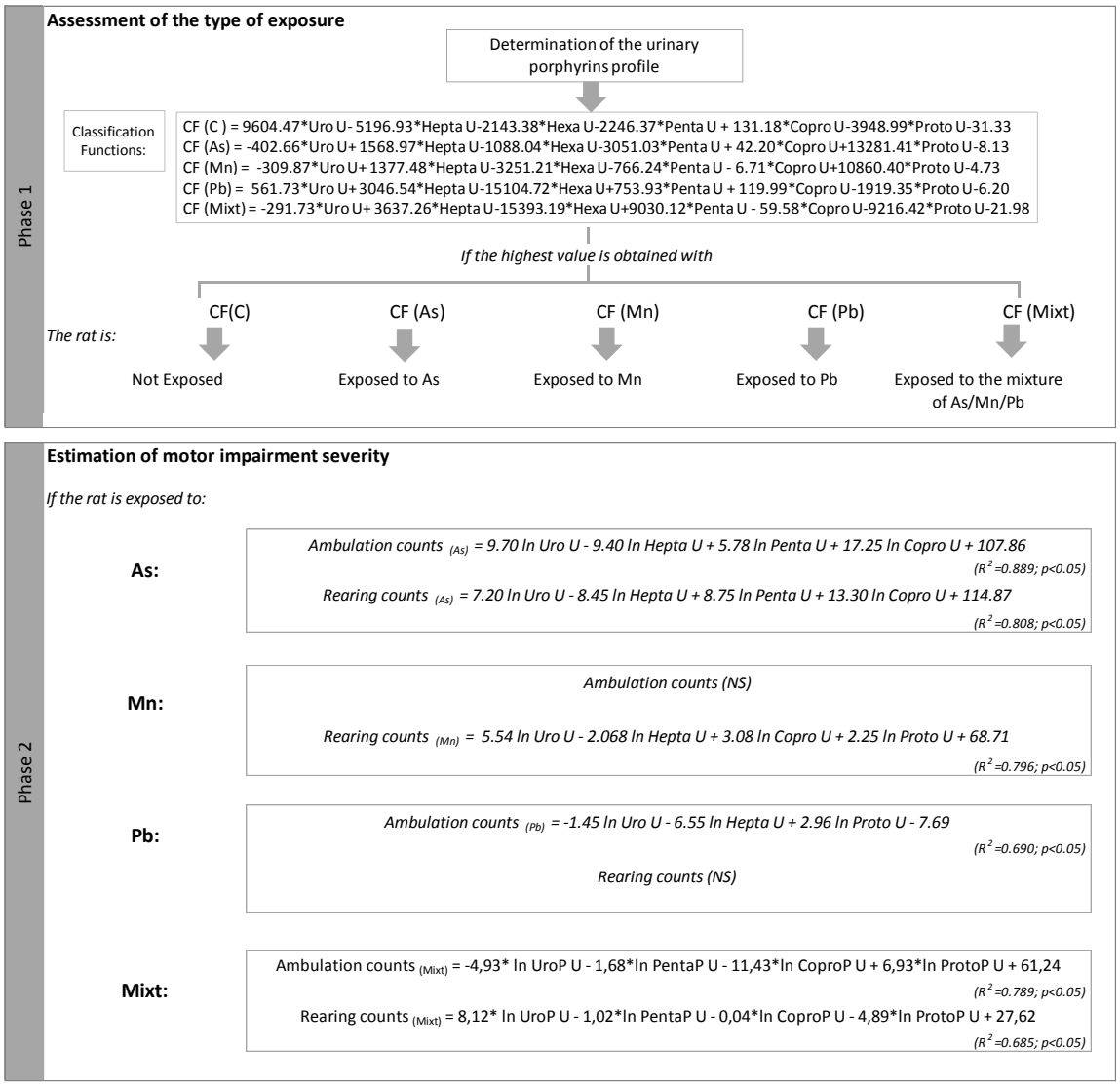


Fig. 5.3: Procedure II proposed the individual assessment of the type of exposure and evaluation of motor activity deficits. Phase 1 allows the identification of the type of exposure of each rat (to Pb, As, Mn or the mixture of these metals) through the determination of the urinary (U) profile, the levels of Uro-, Hepta-, Hexa-, Penta-, Copro- and Protoporphyrins. Five classification functions (CF) were generated: for non exposed rats [CF (C)] and for exposures to Pb [CF (Pb)], As [CF (As)], Mn [CF (Mn)] and to the mixture of the three metals [CF (Mixt)]. The CF with the highest value indicates to the type of exposure predicted by the model. Phase 2 evaluates motor impairment through the estimation of motor activity, ambulation and rearing counts. Depending on the type of exposure, after the combination of porphyrins levels, ambulation and rearing counts of each rat are predicted. R^2 coefficients and significance (p) values of each regression are also represented. NS means "Not significant".

5.3.3. Evaluation of the procedures

5.3.3.1. Integration of biomarkers of exposure (Phase I)

Procedure I

Table 5.3: Identification of the type of exposure (to Pb, As, Mn or the mixture of Pb/As/Mn) of each rat through its levels of Pb and Mn in blood and urinary Pb, As and delta-ALA concentrations, by discriminant analysis. The table represents the type of exposures predicted by the model and the real exposures (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	5	0	1	0	0
As	6	0	6	0	0	0
Mn	6	0	0	6	0	0
Pb	6	0	0	0	6	0
Mixt	6	0	0	0	0	6

96.7% of the cases correctly classified

Discriminant analysis was performed to access if the type of treatment administrated to each rat could be correctly identified through the combination of several BMs, the levels of Pb and Mn in blood and the concentrations of Pb, As and ALA in urine. Table 5.3 shows that the type of exposure of 96.7% of the rats was properly identified. In fact from the whole group of 30 animals, only one was classified erroneously as being exposed to Mn when in reality it was a control rat (Table 5.3).

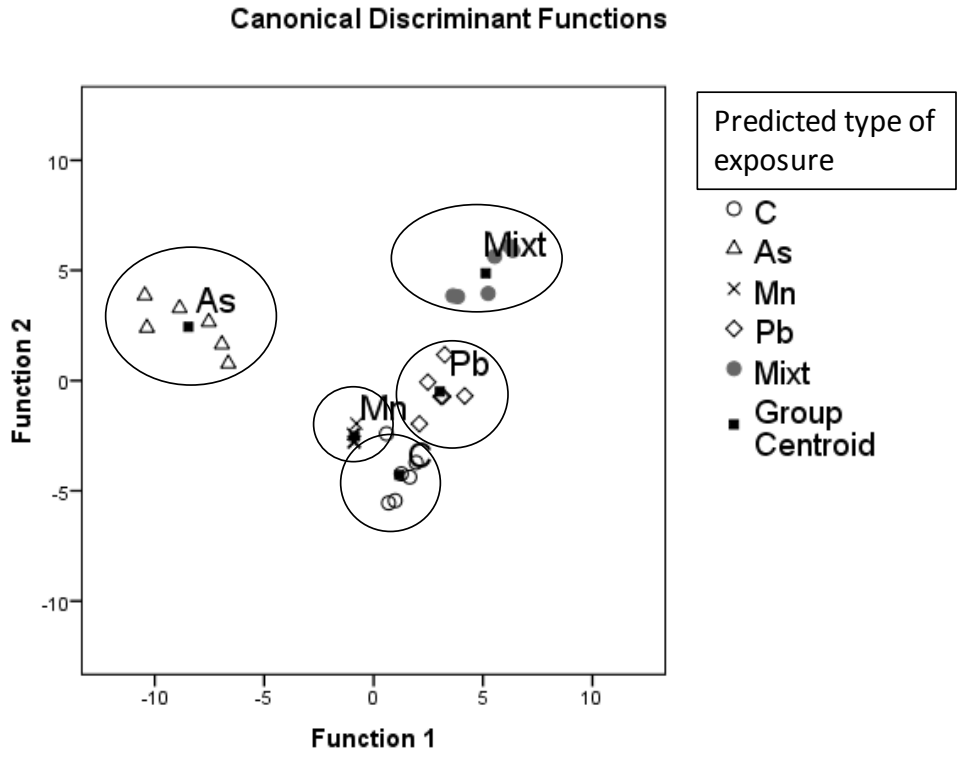


Fig. 5.4: Graphical representation of each rat classification according to the type of exposure, through the integration of its Pb and Mn levels in blood and urinary Pb, As and ALA concentrations by discriminant analysis. A centroid value was calculated for each group and the results are plotted by the canonical discriminant functions.

Fig 5.4 evidences a clear discrimination of Pb, As and mixture treated groups, even despite Pb treated rats were closer to the Mn group and to controls comparing with the other 2 groups. A restricted and well individualized area was attained in the Mn exposed group, however slightly overlapping with controls (Fig 5.4).

Procedure II

Table 5.4: Identification of the type of exposure (to Pb, As, Mn or to the mixture of Pb/As/Mn) of each rat, through its urinary (U) porphyrin profile, the levels of uro-, hepta-, penta-, copro- and protoporphyrins. Discriminant analysis was performed. The table represents the type of exposures predicted by the model and the real exposures (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	6	0	0	0	0
As	6	0	5	1	0	0
Mn	6	0	1	5	0	0
Pb	6	0	0	1	5	0
Mixt	6	0	0	0	0	6

90.0% of the cases correctly classified

Discriminant analysis was performed to evaluate if the type of treatment administrated to each rat could be correctly identified through its urinary porphyrin profile. Table 5.4 shows that 90.0% of the rats were correctly classified. In the Pb exposed group, the classification attributed by the model matched with the administrated treatment in 5 animals, but 1 rat treated with Pb was identified erroneously as exposed to Mn (Table 5.4). Also 5 of 6 rats treated with As were identified as belonging to the As treated group but 1 subject was incorrectly included in the group exposed to Mn, as well as 1 of the 6 rats treated with Mn was wrongly classified as being exposed to As (Table 5.4). Differently, the group (membership) predicted by the model was correct in all the controls and mixture treated rats (Table 5.4).

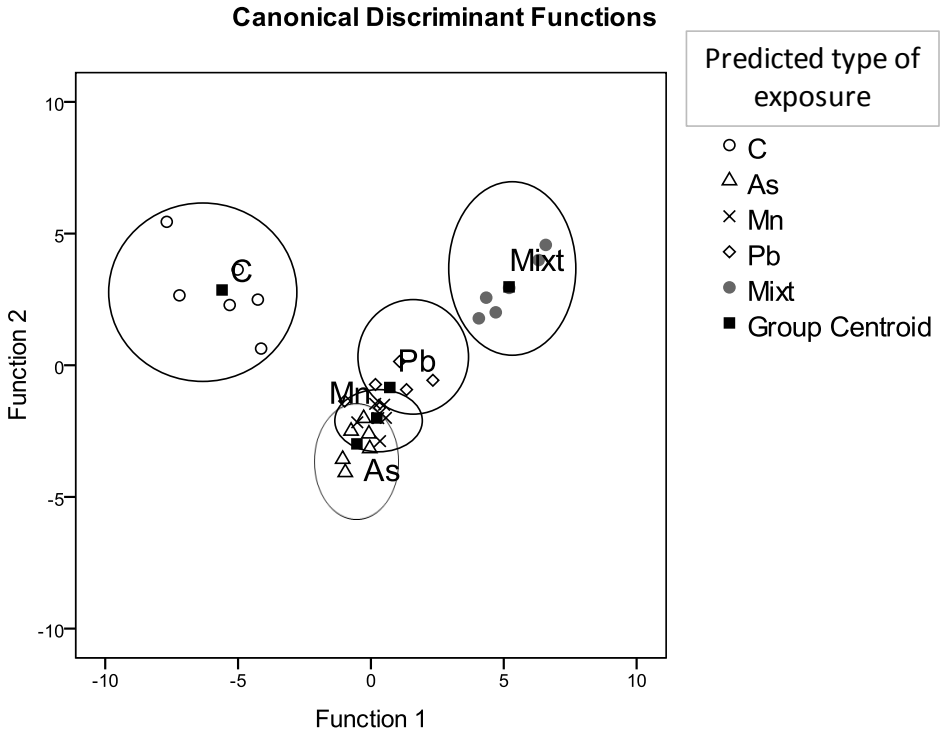


Fig. 5.5: Graphical representation of each rat classification according to the type of exposure, through its urinary porphyrin profile by discriminant analysis. A centroid value was calculated for each group and the results were plotted by the canonical discriminant functions.

The analysis of the graphic in Fig.5.5, allow us to note that both controls and mixture treated rats were clearly separated between them and from all the single exposed groups. On the other hand, there wasn't a clear separation among the three single exposed groups. Mn treated rats' area overlapped with the area corresponding to the As and to the Pb treated animals. Even so, a separation between As and Pb groups was attained (Fig. 5.5).

5.3.3.2. Integration of biomarkers of neurotoxic effects (Phase 2)

Procedure I

Table 5.5: Prediction of motor activity, ambulation and rearing counts of rats exposed to Pb, As, Mn or the mixture of the three metals through the integration of BMs, the levels of Pb, Mn and AChE activity in blood, PRL in serum and urinary levels of Pb, As, ALA and total porphyrins. Data were analyzed by multiple linear regression. All the values estimated by the model (predicted) are compared with the number of movements observed in each rat.

Rat (code number)	Group	Motor activity					
		Ambulation counts			Rearing counts		
		Predicted	Observed	Abs Error	Predicted	Observed	Abs Error
13	As	12	8	4	18	14	4
14	As	9	12	3	16	19	3
15	As	15	20	5	20	28	8
16	As	15	20	5	19	23	4
17	As	8	2	6	17	14	3
18	As	15	12	3	21	13	8
			Average error:	4 ± 1		Average error:	5 ± 2
19	Mn		22		17	14	3
20	Mn		15		14	19	5
21	Mn		30		19	28	9
22	Mn		14		18	23	5
23	Mn		8		16	14	2
24	Mn		6		17	13	4
						Average error:	5 ± 3
25	Pb	25	22	3	28	24	4
26	Pb	18	18	0	21	23	2
27	Pb	11	9	2	6	5	1
28	Pb	22	21	1	31	31	0
29	Pb	22	29	7	22	27	5
30	Pb	21	18	3	25	21	4
			Average error:	3 ± 3		Average error:	3 ± 2
7	Mixt	2	1	1	1	0	1
8	Mixt	3	5	2	8	12	4
9	Mixt	0	2	2	5	5	0
10	Mixt	13	4	9	14	12	2
11	Mixt	8	4	4	9	5	4
12	Mixt	12	7	5	20	7	13
			Average error:	4 ± 3		Average error:	4 ± 4

In Table 5.5, the predicted number of ambulation and rearing movements of each rat were calculated using the formulas presented in Fig. 5.2 and compared with the movements observed after the administration of the last doses of the several metals. In rats treated with Pb, the use of a linear combination of Pb and AChE activity in blood, serum PRL and Pb, delta-ALA and total porphyrins in urine resulted in an average absolute error between predicted and real counts of 3 ± 3 ambulation movements expressed as mean ± sd, which corresponds to 9% of the mean value of ambulation counts observed in controls. An absolute error of 3 ± 2 rearing counts (9% of the mean value of rearing counts observed in controls) was determined (Table 5.5). The motor activity of the rats exposed to As was predicted through the integration of As and total porphyrins in urine and serum PRL, showing prediction deviancies of 4 ± 1 (11%) for ambulation activity and 5 ± 2 (16%) for rearing counts (Table 5.5). The higher errors were detected in the Mn treated group, where using Mn levels in blood, serum PRL and urinary total porphyrins resulted in an average deviancy of 5 ± 3 (16%) in predicting rearing counts, whereas no significant multiregression equation could be obtained to predict ambulation activity. Concerning the group treated with the mixture the errors in predicting motor activity through Pb and Mn blood concentrations and As and total porphyrins in urine were 4 ± 3 (11%) for ambulation and 4 ± 4 (13%) for rearing counts (Table 5.5). More detailed data are presented at Table A3.1 in appendix 3.

Procedure II

Table 5.6: Prediction of motor activity, ambulation and rearing counts, of rats exposed to Pb, As, Mn or the mixture of the three metals through its different urinary porphyrins. Data were analyzed by multiple linear regression. The values estimated by the model (predicted) were compared with the number of movements observed in each rat.

Rat (code number)	Group	Motor activity					
		Ambulation counts			Rearing counts		
		Predicted	Observed	Abs Error	Predicted	Observed	Abs Error
13	As	9	8	1	18	14	4
14	As	13	12	1	21	19	2
15	As	14	20	6	20	28	8
16	As	20	20	0	23	23	0
17	As	6	2	4	15	14	1
18	As	12	12	0	14	13	1
			Average error:	2 ± 3		Average error:	3 ± 3
19	Mn		22		4	14	10
20	Mn		15		4	19	15
21	Mn		30		7	28	21
22	Mn		14		0	23	23
23	Mn		8		0	14	14
24	Mn		6		3	13	10
						Average error:	16 ± 5
25	Pb	19	22	3		24	
26	Pb	19	18	1		23	
27	Pb	15	9	6		5	
28	Pb	17	21	4		31	
29	Pb	30	29	1		27	
30	Pb	20	18	2		21	
			Average error:	3 ± 2			
7	Mixt	3	1	2	6	0	6
8	Mixt	7	5	2	6	12	6
9	Mixt	4	2	2	7	5	2
10	Mixt	2	4	2	5	12	7
11	Mixt	10	4	6	11	5	6
12	Mixt	0	7	7	7	7	0
			Average error:	4 ± 2		Average error:	5 ± 3

The individual estimation of the motor activity of rats exposed to Pb through uro-, hepta and protoporphyrin levels in urine resulted in a prediction average error of 3 ± 2 (9%) for ambulation counts, but no significant regression was obtained to predict rearing movements. In As treated rats the combined urinary levels of uro-, hepta-, penta- and coproporphyrins allowed to predict the ambulation activity with an error of 2 ± 3 (6%) movements and the rearing activity with an error of 3 ± 3 counts (9%) (Table 5.6). Mn treated, rats had only their rearing movements significantly predicted through the integration of uro-, hepta-, copro- and protoporphyrin concentrations in urine, however with an absolute error of 16 ± 5 counts (50%) (Table 5.6). The integration of urinary uro-, penta-, copro- and protoporphyrins in the mixture treated rats predicted motor activity with errors of 4 ± 2 (11%) and 5 ± 3 (16%) respectively for ambulation and rearing counts (Table 5.6). More detailed data are presented at Table A3.2 in appendix 3.

Table 5.7: Prediction of motor activity, ambulation and rearing counts of rats exposed to the mixture of Pb/As/Mn through its brain porphyrin levels, uro-, penta-, copro- and protoporphyrins. Data were analyzed by multiple linear regression. The values estimated by the model (predicted) were compared with the number of movements observed in each rat.

		Motor activity					
		Ambulation counts			Rearing counts		
Rat (code number)	Group	Predicted	Observed	Abs Error	Predicted	Observed	Abs Error
7	Mixt	4	1	3	7	0	7
8	Mixt	6	5	1	9	12	3
9	Mixt	-2	2	4	4	5	1
10	Mixt	9	4	5	13	12	1
11	Mixt	4	4	0	3	5	2
12	Mixt	6	7	1	7	7	0
			Average error:	2 ± 2		Average error:	2 ± 2

Ambulation activity (count) = 21,41* ln UroP Br - 40,42*ln PentaP Br - 8,67*ln CoproP Br - 17,95*ln ProtoP Br- 304,86 (R^2=0,823; p<0.05)

Rearing activity (count) = 17,89* ln UroP Br - 34,46*ln PentaP Br - 13,73*ln CoproP Br - 2,62*ln ProtoP Br- 212,68 (R^2=0,880; p<0.05)

Brain levels of the same urinary porphyrins previously used in the mixture exposed rats to predict their motor activity, were also able to estimate ambulation and rearing counts showing average errors of 2 ± 2 (6%) for ambulations counts and 2 ± 2 (6%) for rearing movements (Table 5.7). No significant regressions were determined using brain porphyrin concentrations, to predict motor activity upon single exposures. More detailed data are presented at Table A3.3 in appendix 3.

5.4. Discussion

Although adverse health effects induced by heavy metals have been known for a long time, exposure to these elements still remains and is even increasing in some areas of the world (Järup, 2003). Pb, As and Mn can occur as mixtures in specific environmental contexts (Dahtrak and Nandi, 2009) being well known the neurotoxic effects induced by these three metals (Sansar et al, 2011; Yadav, 2009; Weiss, 2005). In this perspective BMs have been revealing to be particularly useful to identify exposures and detect induced nervous system progressive diseases, which manifest their symptoms long after exposure to the initiating factor. These markers can be obtained from human tissue and excreta preferentially by noninvasive methods (Kakkar and Jaffery, 2005); blood and urine parameters are already employed to gain information about the CNS in both healthy and diseased states (Mayeux, 2004). Recently it is becoming clear that surveying the several changes in metabolic pathways, using panels of BMs, is more likely to provide a wealth of information that may be difficult to capture by looking only at one pathway or one BM (Kakkar and Jaffery, 2005). The application of statistical models on multiple BM data sets, using discriminant function analysis or multivariate regression techniques, is being used lately to classify groups of interest (e.g., disease or control, pre- or post-drug exposure) and identify disease signatures (Kaddurah-Daouk and Krishnan, 2009). In this work, prediction procedures were designed, through modeling experimental data from rats exposed to Pb, As, Mn

or their mixture, aiming to predict the type of exposure and the magnitude of neurotoxic effects induced in each rat.

5.4.1. Selection of biomarkers of exposure

The ability of each BM to discriminate exposed rats from controls was accessed through ROC analysis. As far as single metal treatments, urinary delta-ALA changes were observed in the Pb treated group and a significant area under a ROC curve (A=100%; $p<0.05$) (Fig.5.1a) was obtained for this BM. Over 99% of Pb present in the blood accumulates in erythrocytes, where more than 80% is bound to ALAD (Ahamed and Siddiqui, 2007); ALAD blood levels may be used as an effect BM, but simultaneously constitute one of the most sensitive indicators of exposure to Pb, largely supplanting the measurement of Pb blood concentrations. Even so, Pb blood concentration is also a reliable BM of exposure to Pb (ASTDR, 2007b) and accordingly exhibited also a significant capability to distinguish Pb treated rats from controls (A=93%; $p<0.05$) but exhibiting an area smaller than blood Pb (Fig.5.1a). Total urinary porphyrins also appeared to be a promising BM of exposure to Pb when considering the significantly increased ($p<0.05$) levels relatively to controls (Fig.4.8, Chapter 4) and the significant ($p<0.05$) areas of 100% under a ROC curve (Fig.5.1a). However similar results were obtained upon As or mixture exposures (Figs.5.1b and d and Fig.4.8, Chapter 4) indicating that the BMs could not distinguish Pb from As exposures. In fact, As urinary levels revealed to be the better BM to identify exposures to As (A=100%; $p<0.05$) (Fig.5.1b and Fig.3.6b, Chapter 3). Urinary elimination is the major route for As excretion, being urinary As measurements more reliable than blood determinations (ASTDR, 2007a; Marchiset-Ferlay et al, 2012) and used as a key BM of exposure to this element (Kakkar and Jaffery, 2005). Serum PRL also increased significantly ($p<0.05$) in this group (Fig.4.6, Chapter 4), but its area under the ROC curve was smaller (A=97%; $p<0.05$) then urinary As' area (Fig.5.1b). Besides, because similar results for serum PRL were attained upon exposure to Mn (Fig.5.1c and Fig.4.6, Chapter 4) the BM reveals to

be unsuitable to distinguish between As and Mn exposures. The observation of Fig. 5.1c indicates that blood Mn (A=100%;$p<0.05$) was the best BM to identify Mn exposures, even with previous studies mentioning that Mn blood levels can only be used on a group basis, not being suitable to be individually used (ASTDR, 2007c; Bader et al, 1999; Cowan, 2008). Mn concentrations in blood also showed to be a better indicator than its urinary level (Santamaria, 2008).

The metal mixture treatment leaded to increased delta-ALA and total porphyrins levels in urine (Figs. 4.7 and 4.8, Chapter 4), with ROC curve analysis revealing that both parameters could reliably differentiate co-exposed rats from controls (A=100%; $p<0.05$) (Fig.5.1d). The increased levels of delta-ALA in urine were higher than the sum of the levels for each of the three single metals treated groups (Fig. 4.7, Chapter 4), suggesting that the mixture treatment leads to urinary delta-ALA changes that exceed the additive effects of the individual metals. Thus, delta-ALA concentration in urine may serve as a better BM of exposure to the metal mixture then total urinary porphyrins. Moreover, despite urinary delta-ALA levels do not distinguish Pb from the mixture exposure, total urinary porphyrins do not allow to differentiate among Pb, As and mixture treated groups.

Considering that metal levels are intrinsically specific to the type of exposure and that urinary delta-ALA concentrations were accurate (A=100%) and less group-unspecific then urinary total porphyrins (Fig.5.1), Pb and Mn blood levels, urinary As and delta-ALA concentrations were selected to be combined to identify the type of exposure.

5.4.2. Selection of biomarkers of neurotoxic effects

The several BMs were tested with respect to their correlation with neurotoxic outcomes induced by Pb, As, Mn or the mixture of the 3 metals. While Pb, As, and Mn metal levels in brain were significantly ($p<0.05$) correlated with decreased motor

activity of each respective single exposed group (Table 5.1), significant correlations were also noted between the three metals brain levels and decreased motor activity in the metal mixture treated group (Table 5.1); this is consistent with studies reporting diminished motor activity in rats co-exposed to Pb/Mn or Pb/As (Chandra et al, 1981; Mejía et al, 1997). However, when pertaining to the feasibility of metal levels in peripheral samples to indicate neurotoxicity, no correlation between Pb blood levels and motor activity was observed possibly due to the low doses used; concerning to Mn, blood Mn was significantly ($p<0.05$) correlated only with rearing counts (Table 5.2), possibly due to the role of different dopaminergic receptors in the mediation of both behaviors (Saldivar-Gonzalez et al, 2009). In fact, only urinary As correlated ($p<0.05$) with both parameters of motor activity (Table 5.2). Respecting to the mixture treated group, despite of Pb and Mn levels in blood and urinary As were significantly ($p<0.05$) correlated with the accumulation of the three metals in the brain (Table 4.2, Chapter 4), no robust associations with motor activity were attained (Table 5.2). Indeed only As urinary levels correlated significantly ($p<0.05$) with both parameters of motor activity and Mn blood levels were merely correlated with statistical significance ($p<0.05$) with rearing counts (Table 5.2). Even so, despite the apparent little value of these BMs to evaluate the neurotoxicity when used alone, the intrinsic role of these parameters to express the presence of the metals, leaded us to select them to be combined in the phase 2 of the prediction models.

Pb, As and Mn interfere with the heme biosynthetic pathway (Ahamed and Siddiqui, 2007; Hift et al, 2011; Wu et al, 2004) and when these events occur, accumulation and increased urinary excretion of delta- ALA and porphyrins can happen (Adhikari et al, 2006; Guolo et al, 1996). When in excess these compounds can act as toxins (Hift et al, 2011; Krishnamurthy et al, 2007) and may be responsible for clinical and pathological pictures observed in porphyrias, which includes central and peripheral nervous system disorders (Kumar, 2012). In Pb treated rats, urinary delta-ALA levels were only significantly correlated ($p<0.05$) with decreased ambulation counts (Table 5.2), maybe because of the low dose used in the experiment. Brain delta-ALA concentrations in the As treated group were significantly correlated ($p<0.05$) with decreased motor activity,

suggesting that the behavioral changes were induced by the As itself (Fig. 4.1, Chapter 4 and Table 5.1) and/or by delta-ALA (Fig. 4.3, Chapter 4). Also increased urinary excretion of delta-ALA in this group correlated with motor activity (Table 5.2) It is also plausible to posit that the decrease in motor activity in Mn treated animals is secondary to the accumulation of Mn in the brain (Table 5.1 and Fig.3.4, Chapter 3). In the mixture treated group, significant correlations ($p<0.05$) were found between motor activity and brain and urinary delta-ALA, suggesting that the accumulation of this compound interfered with behavior performance (Tables 5.1 and 5.2). Concomitantly, urinary total porphyrins correlated significantly ($p<0.05$) with motor activity in As or mixture exposed groups (Table 5.2) as well as in rats treated with the low Pb dose, with respect to ambulation counts (Table 5.2). Some authors defend that porphyrins are promising BMs of toxicity induced by metal mixtures because different metals can inhibit different enzymes of the heme biosynthetic pathway (Woods et al, 2009; Wang and Fowler, 2008). In this work total urinary porphyrins were accumulated in co-exposed rats more than after exposure to each metal alone (Fig.4.8, Chapter 4). These results suggest that heme parameters are promising to be integrated for the evaluation of neurotoxicity in exposed rats.

Serum PRL is becoming increasingly used as a measure of the dopaminergic function in environmental and occupational studies (Meeker et al, 2009; 240, Takser et al, 2004), since PRL release is regulated by the neurotransmitter DA (Marreiha dos Santos et al, 2011). The 3 metals, Pb, As and Mn can interfere with the dopaminergic system, which plays an important role in motor activity, with specifically dopaminergic receptors D1 and D2 involved in the mediation of ambulation, being rearing behavior mediated through D4 receptor (Rodríguez et al, 2010; Saldivar-Gonzalez et al., 2009; Takser et al, 2004). However, when compared with controls, Pb exposed rats had only a slight increase in the serum PRL levels (Fig.4.6, Chapter 4), which were not correlated with ambulation or rearing movements. However, concerning to As treated rats, the significant negative correlation ($p<0.05$) of serum PRL with motor activity justifies the selection of this BM for further integration (Table 5.2 and Fig.4.6, Chapter 4). This is actually an important result given the scarce information about the effects of As on

serum PRL concentrations (Jahan et al, 2012). Mn seems to have selectivity for dopaminergic neurons and, despite controversial, modifications in serum PRL levels have been shown to be induced by Mn (Ellingson et al, 2003). According with this information, increased serum PRL (Fig.4.6, Chapter 4) correlated significantly ($p<0.05$) with the motor activity of Mn treated rats (Table 5.2). In the mixture treated rats, Pb was the metal with the higher concentration in the brain (Fig.3.4, Chapter 3), and probably its limited influence on serum PRL, counteracted As and Mn effects on this hormone (Fig.4.6, Chapter 4). This final effect might explain the absence of correlation between serum PRL and motor activity of co-treated rats, which decreased more markedly than after single exposures (Fig. 3.2, Chapter 3). Also, and according with our results, we posit that As and Mn may contribute more than Pb to impair motor activity via dopaminergic pathway dysfunction; it seems that in the mixture exposed group, motor activity dysfunction occurred via multi-mechanisms leading to such a complex figure. Considering the interpretation of our results, serum PRL levels were also selected to be tested in the phase 2 of the model.

Regardless the involvement of the cholinergic system in muscular contraction and locomotion (Bowler et al, 2006) the dopaminergic system plays a prominent role in the modulation of motor activity (Bardullas et al, 2009). No correlations of AChE activity in brain with levels of metals in this organ or motor activity were observed in the single treated groups. Differently, in co-treated rats, AChE brain levels correlated significantly ($p<0.05$) with motor activity (Table 5.1), but this correlation had not occurred with its blood concentrations. It is plausible that AChE activity alterations did not relevantly contributed to the observed motor dysfunctions in single exposed groups; The activity of this enzyme in the blood does not seem to be suitable to indicate the neurotoxicity induced by Pb, As, Mn or its mixture.

5.4.3. Using single selected biomarkers to identify exposure and predict the magnitude of neurotoxic effects

Delta-ALA and total porphyrins levels in urine showed to be accurate BMs of exposure to Pb, but not exclusively for this group (Figs.5.1a, b and d); Pb blood levels were less accurate then urinary levels (Fig.5.1a). Differently, urinary As exhibited accuracy to access exposure only for the As treated group (Fig.5.1b), Nevertheless indicates neurotoxicity also for mixture treated group (Table 5.2). Mn levels in blood showed to be a more accurate BM than serum PRL to access Mn exposure (Fig.5.1c) and only for the Mn treated group. However, Mn blood levels correlated significantly ($p<0.05$) only with ambulation counts (Table 5.2). The PRL levels in serum were found to be promising as a BM of neurotoxicity induced by Mn, requesting further studies with DA measurements in the brain. Both urinary delta-ALA and total porphyrins showed to be accurate BMs of exposure in rats exposed to Pb, As or the mixture of Pb/As/Mn and not only for one group (Figs.5.1a, b and d). Delta-ALA levels revealed to be reliable BMs of neurotoxicity induced by this mixture but also significantly ($p<0.05$) correlated with motor activity in the Pb treated group. These parameters were not exclusive for the mixture exposed group (Table A2.1, appendix 2)

In general the investigated BMs were not satisfactory when used alone, regarding to the following compromise: accuracy to distinguish among Pb, As, Mn and Pb/As/Mn mixture treated rats; to indicate neurotoxicity; and to be exclusive of only one group. Moreover respecting to neurotoxicity, they were applied only in a group basis. In addition, and concerning to brain porphyrins or their urinary profile, no porphyrin alone correlated with motor activity of all the exposed groups. The obtained results points towards the pertinence of testing them in combination.

5.4.4. Integrating biomarkers

Two procedures were generated and compared between them, as well as with single BMs methodologies. In both, the peripheral BMs were integrated to generate a 2 phase's prediction model, with phase 1 aiming to identify the type of exposure of each rat, and phase 2 aiming to subsequently predict the magnitude of motor disarrays. In procedure I the levels of the 3 metals in blood and/or urine, urinary ALA and total porphyrins, blood AChE activity and serum PRL were the integrated BMs, whereas procedure II combined several urinary porphyrins.

5.4.4.1. Identification of the type of exposure

Concerning to the phase 1 of both procedures, discriminant analysis was performed after testing several combinations of BMs, which were used to generate 5 classification functions, corresponding each function to one type of exposure: not exposed (controls), exposed to Pb, As, Mn or their mixture (Figs.5.2 and 5.3). Regarding to procedure I, it is not surprising that the inclusion of Pb, As and Mn levels in the model resulted in a good discrimination among groups, since the detection of metals in biological samples is the most common indication of their exposure (Phoon, 1998) and body burden is generally determined using blood or urinary concentrations (Kakkar and Jaffery, 2005). Fig 5.4, illustrates that both Pb and As groups formed separated and well defined clusters, did not overlapped with controls and plus the type of exposure of all Pb or As treated rats was identified correctly (Table 5.3). Even so, Pb treated rats were closer to controls than As or mixture treated animals (Fig.5.4), possibly because the Pb low dose administrated in the experiment (Fig. 3.1, Chapter 3) did not allow a marked detachment from non-exposed rats. In fact, it is truth that urinary Pb levels have been used to assess Pb exposure, but only on a group basis (Fukui et al, 1999). This low Pb dose might explain the need of using both blood and urinary Pb levels in the classification functions (Fig.5.2). The Mn treated group exhibited the worst discrimination, with a poor separation from controls (Fig. 5.4); in addition one control

rat was categorized incorrectly as being exposed to Mn (Table 5.3). Accurate BMs of exposure to Mn are still lacking and to date it is generally achieved that blood and urine levels can be used to confirm exposure, but they are not suitable to be used on an individual basis (ASTDR, 2007c; Bader et al, 1999; Cowan, 2008), while we aimed to assess exposures individually. Certainly integrating blood Mn levels in the classification functions (Fig.5.2) improved the model just because this weak BM contributed together with others to predict the exposure. There is a need of better BMs of exposure to Mn to be integrated in these models. The mixture treated rats formed a well-defined cluster which was clearly separated from all the other groups (Fig. 5.4). Moreover, the type of exposure predicted by the model was correct for all the mixture exposed rats (Table 5.3). Indeed, the presence of the three metals is represented in the classification functions (Fig.5.2) and urinary delta-ALA changes plausibly played a relevant role on the discrimination of co-exposed rats. In fact, the urinary excretion of delta-ALA in co-treated rats exceeded the additive effects of the individual metals (Fig.4.7, Chapter 4) and the previous results suggest that this BM alone could serve a sensitive peripheral BM of exposure to the mixture (Andrade et al, 2013).

The urinary porphyrin profile was also integrated to differentiate among exposures to Pb, As, Mn or the mixture of Pb/As/Mn (procedure II) and allowed to distinguish between Pb and As exposed groups, when considering that no wrong classifications (Table 5.4) neither an overlap occurred between these groups (Fig.5.5). Actually, significant differences ($p<0.05$) between these groups were attained in the porphyrin profile, regarding to uro- and protoporphyrins (Fig.4.9a and f, Chapter 4). However, in Fig.5.5 the values of Pb and As groups were closer, possibly due to the inhibition of a common enzyme, coproporphyrinogen oxidase (Ahamed and Siddiqui, 2007; Ng et al, 2005). Indeed, a similar magnitude of increased coproporphyrin excretion, as compared with controls, was noted in both exposed groups (Fig.4.9e, Chapter 4). The integration of the urinary porphyrins profile barely distinguished Mn exposure from exposures to Pb or As (Fig.5.5), with 2 rats treated with Mn wrongly classified, one as fitting in the As treated group and another as belonging to the Pb treated group (Table 5.4). The most relevant change observed in Mn treated rats was a pronounced

decrease in coproporphyrins, which is a porphyrin also affected by Pb and As. Differently, the profile of the heme precursors in urine differentiated all the mixture exposed rats not only from controls but also from all single treated animals. The treatment administrated to every co-exposed rat was identified correctly (Table 5.4) and a clear separation of this group from all the other groups is revealed in Fig.5.5. Indeed hexa-, penta- and coprophorphyrin levels in this group were significantly ($p<0.05$) increased as compared with all the other groups (Fig.4.9c, d and e, Chapter 4) as well as uroporphyrins were significantly ($p<0.05$) decreased with respect to the other groups, excepting the Pb treated group (Fig.4.9a, Chapter 4). These results are in agreement with authors that indicate porphyrins as promisors BMs of mixtures of metals (Wang and Fowler, 2008).

5.4.4.2. Predicting the magnitude of neurotoxic effects

In both procedures (I and II), phase 2 was applied aiming to predict the severity of induced neurotoxic effects in each rat. Being motor activity in open-field a behavioral test accepted as sensitive, reliable and efficient to evaluate neurotoxicity (Santos, 2008), this endpoint was evaluated 24 h after the administration of the last dose to the 5 groups, through the determination of ambulation and rearing counts. Using multiregression analysis, linear combinations of BMs were used to estimate, in each exposed group, the number of ambulation and rearing movements of each rat.

In procedure I while the same BMs were applied in different exposures, others BMs were exclusive of one group (Fig.5.2). Respecting to BMs used in more than one type of exposure, the inclusion of each metal level in the multi-regression equations revealed useful to estimate the motor activity in all the single exposed groups (Fig. 5.2); for the co-treated rats the combination of the three metal levels, urinary As and Pb and blood Pb and Mn levels, improved the prediction capability of the model (Fig.5.2), even when not all the peripheral metal concentrations correlated with behavior (Table 5.2). It seems that the integration of weak BMs results in a global

enhanced performance. Despite not all the correlations between total urinary porphyrins and motor activity in single exposed groups were significant, urinary total porphyrins revealed to be useful when present in the multiregression equations of all the groups as an X variable (Fig.5.2). The significant ($p<0.05$) increase of total porphyrins in urine observed in Mn exposed rats (Fig.4.8, Chapter 4) did not correlate with motor activity (Table 5.2), but paradoxically improved the prediction of rearing movements by multiregression, certainly demanding further explanation. Along with the concentrations of the three metals, it was only necessary to include total porphyrins in urine in the regression equations to significantly ($p<0.05$) estimate the motor activity in co-treated rats (Fig.5.2). The integration of urinary delta-ALA levels instead of the total porphyrins leaded to similar outcomes (not shown results), however slight better predictions were achieved using total porphyrins.

Serum PRL was used in multi-regression equations to predict motor activity in all single exposed groups (Fig. 5.2) including Pb, despite the previous apparent unsuitability of this BM to indicate neurotoxicity upon Pb exposure (Fig. 4.6, Chapter 4). Also blood AChE activity was integrated to predict the motor activity of Pb treated rats (Fig.5.2), when the mean values of this parameter were actually different from controls, but no statistical difference was attained due to the huge magnitude of the standard deviation values (Fig.4.5, Chapter 4). In truth, given the low Pb administrated doses, the best fit (R^2) of the multi-regression equations (which moreover was not statistically significant) was only attained when all the available BMs were integrated (Fig.5.2). Again, possibly weak BMs already excluded under traditional single BM approach interpretations may effectively have some value when combined with others. Moreover, a BM considered weak on a group basis, should not necessarily be rejected if destined to be applied integrated in an individual basis.

In Procedure II, the integration of urinary porphyrins leaded to observe several significant ($p<0.05$) multi-regression equations estimating the motor activity of single exposed groups (Fig.5.3). However no significant regressions with ambulation or

rearing counts were obtained when the levels of the same porphyrins in the brain were integrated. Thus, the presented data regarding to rats treated with only one metal should be considered with some reservations.

In co-treated rats there was a trend for a decrease in motor activity higher than after single exposures (Fig.3.2a and b, Chapter 3), but this alteration did not correlate with the levels of each brain porphyrin; this alterations were not evinced enough, possibly due to brain regulation mechanisms of porphyrin levels and to the use of low to moderate metal doses in this experiment. Remarkably, when porphyrins that only exhibited slight modifications in the brain (uro-, penta-, copro- and protoporphyrins) (Fig.4.4a, d, e and f, Chapter 4) were taken together through linear combination, they predicted significantly ($p<0.05$) the values of motor parameters in the rats (Table 5.7). These results suggest a relevant role of porphyrins on neurotoxicity induced by the co-exposure to the mixture of Pb, As and Mn. In addition, porphyrins for which some alterations were observed in the brain were also changed in the urine (Figs.4.4 and 4.9, Chapter 4), suggesting that the integration of the urinary porphyrin profile may express modifications in brain porphyrin levels. This is a meaningful outcome when considering the purpose of using peripheral porphyrin levels to detect changes in such an inaccessible tissue in humans. Indeed the linear combination of the urinary levels of these porphyrins (uro-, penta-, copro- and protoporphyrins) also estimated significantly ($p<0.05$) the number of ambulation and rearing counts (Table 5.7). This work shows that both brain and urine porphyrins can predict the magnitude of motor alterations in the co-treated rats.

For both procedures all the integrated BMs were tested with respect to their capability to predict exposures and motor activity when applied alone (Tables A1.1-15, appendix 1 and Tables A2.1-6, appendix 2). However, none BMs alone had the capability to distinguish the 5 groups of rats with a % of wrong cases lower than after its integration (Tables A1.1-15, appendix 1). As far as motor activity is concerned, some significant correlations were obtained with single BMs, but apart from metal levels, they were not

exclusive of one type of single exposure (Table A2.1, appendix 2). Actually the urinary levels of hexaporphyrins were group specific for co-treated rats and a significantly ($p<0.05$) regression with motor activity was observed (Table A2.1, appendix 2), but again with general higher errors in predicting motor activity then after using multi-regression equations (Table A2.6, appendix 2). When using the levels of metals alone the errors were generally higher than upon their integration (Tables A2.2- 5, appendix 2).

5.5. Conclusions

In phase 1, the combination of urinary Pb, As, delta-ALA and blood Pb and Mn levels identified the type of exposure (procedure I) better than the integration of urinary porphyrins (procedure II), as 97% versus 90 % of cases were classified correctly. Even so, despite the use of the urinary porphyrin profile resulted in 10% of wrong cases, such practical alternative should not be rejected to be further studied. Concerning the prediction of motor disarrays (phase 2) due to the absence of correlation of brain porphyrins with motor activity some reserve is attributed to the integration of these heme precursors (Procedure II) to predict the magnitude of motor disarrays upon single exposures. Differently the combination of metal levels, urinary total porphyrins, serum PRL and/or blood AChE activity (procedure I) seemed to be a valid approach for single exposed subjects. On the other hand, both procedures (I and II) predicted the magnitude of motor impairments induced by the mixture of Pb, As and Mn in each rat. The combination of urinary porphyrins resulted in slight lower errors and offers the possibility of using an exclusively safe and non-invasive method of urine sample collection and the determination of the several BMs by a single and quick HPLC analysis. It is also suggested that weak and/or unspecific BMs can gain global strength when combined with others.

Generally the combination of exposure and/or effect BMs in rats exposed to Pb, As, Mn or this metal mixture allows a correct identification of the type of exposure as well as a reasonable evaluation of motor activity impairments of individual subjects. Therefore, the integration of BMs revealed to be a better predictive tool than the traditional approach focused on the search of a strong BM to be used alone. The outcomes arising from this work raises the possibility that exposures can be identified and that the severity of neurotoxic effects induced by this mixture can be semi-quantitatively predicted in humans, opening a door for the chance of detection of neurotoxic outcomes in a pre-clinical and still potentially reversible stage. The research on BMs integration methodologies seems certainly promising to assess “real-life” scenarios of exposure to chemical mixtures.

Chapter 6

Preliminary study in a Portuguese risky population: biomonitoring miners co-exposed to lead, arsenic and manganese

6.1. Background

Humans are environmentally exposed to a range of chemicals, through air, water, soil and food, all contributing to a complex exposure in daily life (Kossowska et al, 2013). In addition, in several occupations other types of exposure occur during the performance of job duties and actually, for centuries, the work environment has significantly contributed to an increased risk of adverse health effects due to chemical hazards (Casarett and Doull's, 2007). In both situations, exposure to metals are frequently related to the development of toxicity and pathological conditions (Farina et al, 2012) and even low level metal exposures may induce long-term neurological health risks (Christensen, 1995), which are increasingly worrying during the last decades (Kossowska et al, 2013; Meyer-Baron et al, 2012). Most frequently, heavy metals occur as mixtures (Farina et al, 2012) and mixed exposures may have a large impact on human health since the induced effects may be higher than simply adding the effects of the individual components (Kossowska et al, 2013).

6.1.1. Exposure to metals in urban environments

In the cities, people may be exposed to more toxic elements via ingestion and inhalation of contaminated particulates, compared with rural or non-industrialized areas (Huang et al, 2014). The problem of atmospheric contamination by air-borne particulate matter containing heavy metals has notably worsened in the last few years due to the increase in motor vehicles, urban constructions, heat installations and industry (Cavanagh et al, 2009; Fernandez et al 2000). Moreover, urban areas are the most densely populated regions of the world because of their strong industrial and economic activities. After abolition of leaded gasoline, decreased Pb levels in the air and in blood of exposed populations were detected in several world regions (ASTDR, 2007b). Yet, Pb is still a major source of pollution in urban soils (Cheng et al, 2014) and studies performed on the characterization of atmospheric particulates and particle-

bound transition metals in some cities (e.g. in the center of Athens, Greece) indicated that Pb was among the largest components in various airborne particulate matter fractions (Valavanidis et al, 2006). The accumulation of As in urban environments is most often attributed to fossil fuel combustion, particularly in coal, metal-processing industries and mining activities (Cheng et al, 2014). Areas with high environmental As concentration include certain regions in Australia,, Indonesia, Italy and an electronic waste recycling area of a China province (Huang et al, 2014). Bangladesh is grappling with the largest mass poisoning of a population in history because ground-water used for drinking has been contaminated with naturally occurring inorganic As (Smith et al, 2000). Arsenic was also found among the most abundant metals present in water-soluble extracts of particulate matter in Christchurch and Auckland, in New Zeeland (Cavanagh et al, 2009) and water contaminations with As were also detected near Panasqueira, Lousal and S. Domingos mines, in Portugal (Grangeia et al, 2011; Ferreira da Silva et al, 2009a and Pereira et al, 2004). With respect to Mn, human exposure from natural and anthropogenic activities may arise from the release of this metal into waterways through the erosion of rocks and soils, mining activities and industrial waste, such as dry-cell batteries. Almost 80% of industrial emissions of Mn are attributable to iron and steel production facilities (ASTDR, 2007c). Also due to the use of methylcyclopentadienyl manganese tricarbonyl (MMT) as an additive in gasoline in countries like Argentina, Australia, Bulgaria, Canada and Russia, atmospheric Mn concentrations are increasing (Bolté et al, 2004; Gulson et al, 2006); indeed, elevated levels of this metal in children's blood have been detected in regions where the additive is allowed (Batterman et al, 2001; Gulson et al, 2006). When considering that there are no frontiers for pollution, Mn exposure to general population may become a relevant matter in a near future.

Therefore, complex co-contaminations of trace metals exist in many cities, with Pb, As and Mn as their components, while great variations from one city to another may exist, depending on the type of major industries in the urban area. To illustrate in a study of the metal content of the urban soil of 31 cities in China, it was observed that among the 10 metals exhibiting the highest levels, Pb occupied the 5th position and As

the 8th (Cheng et al, 2014). Scalp hair samples collected from Guangzhou (China) urban population, showed significant higher levels of Pb, As and Mn as compared with a non-exposed population, in the same way as previously reported in other studies in Karachi, Pakistan (Huang et al, 2014). Metals are also an important component of urban particulate matter in Mexico City (Mexico), with children living in this area exhibiting accumulation of ultrafine metal containing particulate matter in the nasal respiratory tract, that included Pb, As and Mn. Brain inflammation, cognitive deficits and brain magnetic resonance imaging (MRI) structural abnormalities were also detected, leading the authors to express their concern with respect to "interactions" or "joint toxic actions" among metals (Calderón-Garcidueñas et al, 2013).

6.1.2. Exposure to metals in occupational settings

Millions of workers are employed in manufacturing, mining, construction and other industries where significant amounts of airborne metals and metal compounds are generated (Ashley, 2009). The particles produced in these milieus are of major concern when compared with particulate in outdoor environment due to :i) higher concentrations inside industries; ii) the particles may contain more toxic compounds and in higher concentrations; iii) the majority of the people spend more time in the work-place (33% of the day) than in the outdoor. Therefore, exposure to pollutants in the workplace is more relevant than outdoor pollution and despite the technological requirements imposed by safety regulations, workers continue to be excessively exposed to dangerous chemicals, not only due to the ineffectiveness or inexistence of equipment that promotes the extraction of particles or individually protects the workers, but also due to the negligence of the subject himself (Félix et al, 2013). The variety of metals to which workers can be exposed may cause occupational illness (Ashley, 2009; Félix et al, 2013) and although occupational settings are nowadays safer , efforts still exist towards the recognition of the causal link of exposures to chronic diseases and/or diseases with long latencies

(Casarett and Doull's, 2007), such as the neurotoxic outcomes (Ashley, 2009). Since the late 2000s, neurological disorders from long term low level exposures became a major topic and with the aim of detecting earlier changes in neurological functioning in workers exposed to neurotoxic chemicals, several studies have been and are still conducted (Meyer-Baron et al, 2012). With respect to miners, the most commonly studied occupational health problems are silicosis, coal workers' pneumoconiosis, asbestosis and other diseases, but there are very few studies that correlate adverse health effects with the exposure of miners to toxicants present in their work environment. Pb, As, Mn and Pb co-occur in occupational settings like mines (Dahtrak and Nandi, 2009), and may provoke adverse health effects in the workers, including the neurological, contributing to reduce economic productivity (Landrigan et al, 2006). Hence, biomonitorizing mine workers for low toxic levels of neurotoxic metal mixtures is surely indispensable (Dahtrak and Nandi, 2009). In this perspective, occupational toxicology is focused on the application of toxicology principles and methodologies toward chemical hazards encountered at work, aiming to prevent and control occupational disease using BM as important tools (Casarett and Doull's, 2007; Schulte and Hauser, 2012). However, nowadays additional challenges are recognized since the changing pattern of human exposure to a more complex low level exposure requires improvements in the capacity to identify and monitor subgroups, using model predictions for the estimation of exposures and for diagnostic purposes (Kossowska et al, 2013). Additionally, as trace elements concentration in humans is affected by physiological parameters and genetic traits, it may vary considerably among subgroups and even among subjects. When workers are exposed to a mixture of trace elements, a single BM is rarely sufficient to classify the exposure or quantitatively predict the outcomes of one single person. Therefore, a new approach for dealing with combined exposures and the inherent possibility of interferences among various elements, is the acquisition of information from multivariate analysis of different parameters measured simultaneously in the same sample (Christensen, 1995).

A reasonable number of works indicate that several mine surrounding areas in Portugal are contaminated with Pb, As and/or Mn (Carvalho et al, 2013; Grangeia et al, 2011; Pratas et al, 2013). Despite the existence of little information about the levels of occupational exposure to these metals or its mixtures, it is the opinion of some authors that Portuguese mining activities may adversely affect the health of miners (Coelho et al, 2013). Being the use of animal models a relevant tool for the prediction of human safety (Chapman et al, 2013), previously an in vivo assay was performed using Wistar rats, which were exposed to Pb, As, Mn, or the mixture of these metals (Fig.3.1, Chapter 3). The levels of the metals and several other BMs of exposure and/or predictive of neurotoxic effects were determined and later, selected BMs were combined to identify the type of exposure aiming a further application in a population of Portuguese workers exposed to the same mixture. Preliminary data concerning to the levels of exposure to a mixture of Pb, As, Mn were collected in a Portuguese population occupationally exposed, miners from Alentejo. Two control populations, not occupationally exposed, were used: one belonging to the administrative service of the mining company and living in a rural area and the other living in an urban area of Portugal, Lisbon.

6.2. Methodology

6.2.1. Chemicals

Chemicals were obtained from the following source: sodium borohydride ($NaBH_4$; >98%), ascorbic acid ($C_6H_8O_6$; >99%), potassium iodide (KI; >99%) and sodium hydroxide reagent grade (NaOH; > 98%) from Sigma.

6.2.2. Experimental design

Biological samples of workers from a mining company in Alentejo (Portugal) (group M) were obtained from the Occupational Health department under the supervision of the medical doctor of this company. Two non-exposed groups were used in this study: one with people not occupationally exposed in mines and living in an urban area (Lisbon, Portugal), obtained from the laboratory of Clinical Analysis of the Faculdade de Farmácia, Universidade de Lisboa (group CU) and the other constituted by the administrative workers of the mining industry in the rural area of Alentejo (group CR). All the samples correspond to male adult persons.

6.2.3. Sample collection

All the biological samples, blood and urine, were collected on the last day of the shift and stored at –80°C until the analysis. The blood was stored in heparinized tubes, and the urine samples were collected in 25-mL aseptic containers. The collection of all the samples was performed under the supervision of a qualified nurse. Twenty samples were used for each group.

6.2.4. Determination of biomarkers

The levels of Pb and Mn in blood and urine, AChE activity in blood and urinary delta-ALA, total porphyrins and the porphyrin profile were determined as previously described in Chapters 3 and 4. Concerning human blood and urine As analysis it was expected lower levels of As than in treated rats, thus, we used the hydride generation atomic absorption spectrometry (HGAAS), which would allow us to detect lower concentrations of As.

6.2.4.1. Arsenic analysis by hydride generation atomic absorption spectrometry

The levels of As were determined by HGAAS. In this technique, a sample loop on a flow injection valve is filled with an acidified sample, blank, or standard. The sample is mixed with a pumped stream of sodium borohydride, to produce arsenic hydrides in vapor phase. A flow of argon is added to this mixture allowing the gaseous phase containing the analyte to enter the quartz cell on the AAS, for analysis (Hineman, 2012).

Prior to analysis the samples were digested as described in Chapter 3 and then acidified through the following procedure: to 1 mL of each sample, 1 mL of HCl, 1 mL of ascorbic acid 31% (w/v) and 1 mL of potassium iodide 31% (w/v) was added and filled in a volumetric flask to 10 mL with deionized water. After 45 minutes at room temperature, total As determination was carried out using a PerkinElmer AAnalyst™ 700 equipped with a hydride generation system, Perkin-Elmer Corp. Hydride generation was performed using 0.2% (w/v) $NaBH_4$ in 0.05% NaOH and 10% HCl. The radiation source was a hollow cathode lamp of As (Perkin-Elmer) used at a wavelength of 193.7 nm and a spectral slit width of 0.7 nm. The QL of the method was 0.037µg/L.

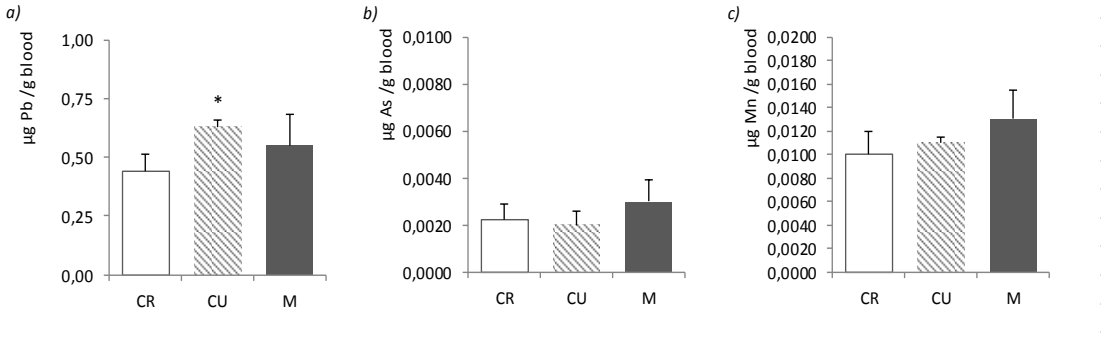
6.2.5. Statistical analysis

All the groups were compared by Mann-Whitney tests and BMs were integrated using discriminant analysis and multi-regression statistical tools, as previously described in Chapters 3 and 5.

6.3. Results

6.3.1. Biomarkers in blood

Metal levels



Figs.6.1. (a), (b) and (c): Blood concentrations of Pb (a), As (b) and Mn (c) in rural (CR), urban (CU) and mining (M) populations. Data represent the mean ± SD and n=15 each group. All the groups were compared by Mann-Whitney U tests. * and ^ are $p < 0.05$ versus CR and CU.

Slight increases of Pb, As and Mn were attained in the blood of miners, with respect to the rural group, however none of these differences were statistically significant (Figs.6.1a, b and c). Pb concentrations in the blood of the urban population were significantly ($p<0.05$) higher than in the rural group (Fig.6.1a).

AChE activity

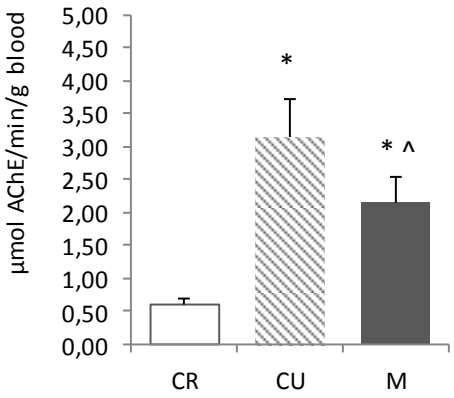
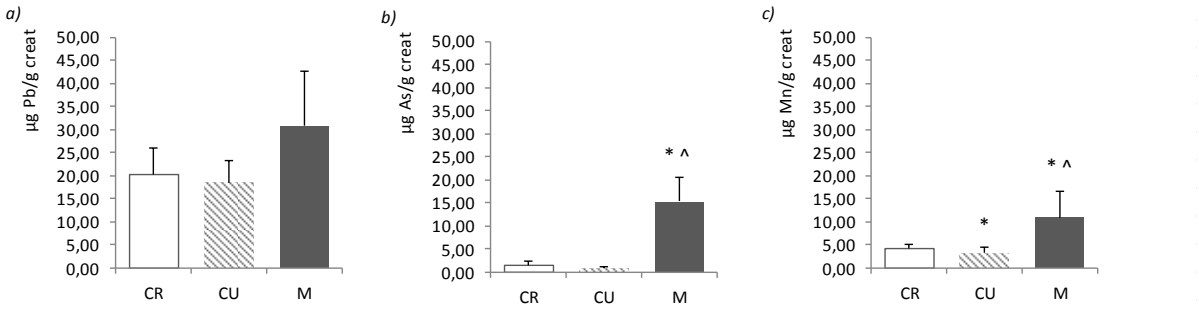


Fig.6.2: Total blood AChE activity in rural (CR), urban (CU) and mining (M) populations. Data represent the mean ± SD and n=15 each group. All the groups were compared by Mann-Whitney U tests. * and ^ are $p < 0.05$ versus CR and CU.

AChE activity in the blood significantly ($p<0.05$) increased in both urban and mining populations comparing with the rural group (Fig. 6.2). The urban group exhibited the highest levels of the parameter, which was significantly ($p<0.05$) higher than the value observed in the miners (Fig. 6.2).

6.3.2. Biomarkers in urine

Metal levels



Figs. 6.3 (a), (b) and (c): Urinary concentrations of Pb (a), As (b) and Mn (c) of rural (CR), urban (CU) and mining (M) populations. Data represent the mean ± SD and n=20 each group. All the groups were compared by Mann-Whitney U tests. * and ^ are $p < 0.05$ versus CR and CU.

The miners´ Pb, As and Mn urinary levels were higher than in rural and urban groups (Figs.6.3b and c), although Pb concentrations lacked statistical significance ($p>0.05$) (Fig.6.3). Mn urinary levels of the urban population were significantly ($p<0.05$) lower than the rural group (Fig.6.3c).

Delta-ALA levels

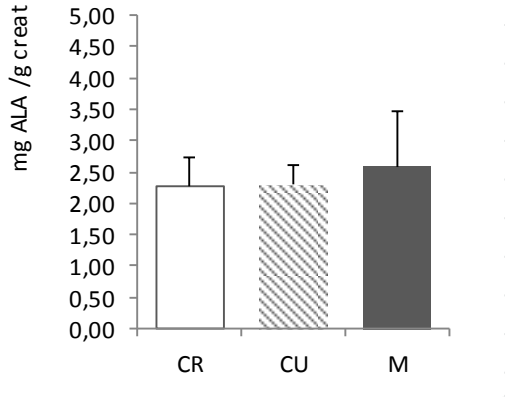


Fig.6.4: Urinary concentrations of delta-ALA in rural (CR), urban (CU) and mining (M) populations. Data represent the mean ± SD and n=20 each group. All the groups were compared by Mann-Whitney U tests. * and ^ are $p < 0.05$ versus CR and CU.

No significant differences were attained among groups respecting to urinary delta-ALA concentrations.

Total porphyrins

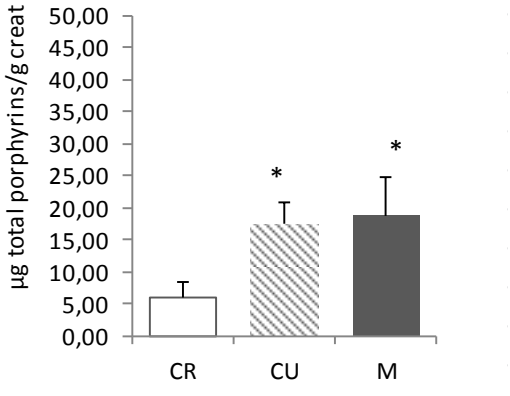
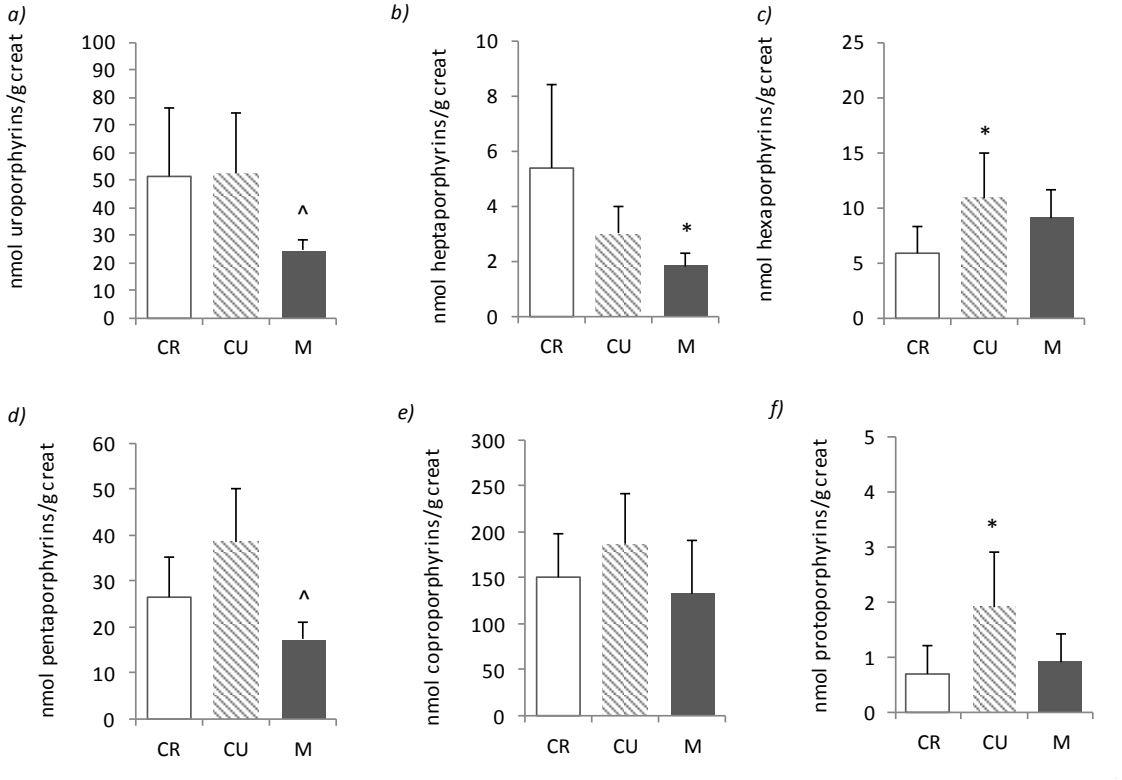


Fig.6.5: Urinary concentrations of total porphyrins of rural (CR), urban (CU) and mining (M) populations. Data represent the mean ± SD and n=20 in each group. All the groups were compared by Mann-Whitney U tests. * and ^ are $p < 0.05$ versus CR and CU.

The concentrations of total porphyrins in the urine of miners and urban subjects were significantly ($p<0.05$) higher than the ones determined in the rural group.

Porphyrin profile



Figs. 6.6 (a), (b), (c), (d), (e) and (f): Urinary levels of uroporphyrin (a), heptaporphyrin (b), hexaporphyrin (c), pentaporphyrin (d), coproporphyrin (e) and protoporphyrin (f) in rural (CR), urban (CU) and mining (M) populations. Data represent the mean ± SD and n=20 each group. All the groups were compared by Mann-Whitney U tests. * and ^ are $p < 0.05$ versus CR and CU.

The miners group revealed to have urinary levels of uro- and pentaporphyrins significantly ($p<0.05$) lower than the urban group (Fig.6.6a and d) and heptaporphyrin excretion significantly ($p<0.05$) lower than the rural subjects (Fig.6.6b). Urban subjects exhibited urinary hexa- and protoporphyrins concentrations significantly ($p<0.05$) increased as compared with the rural group (Fig.6.6c and f).

6.3.3. Integration of biomarkers

Assessment of the exposure in miners, urban and rural subjects (Procedure I')

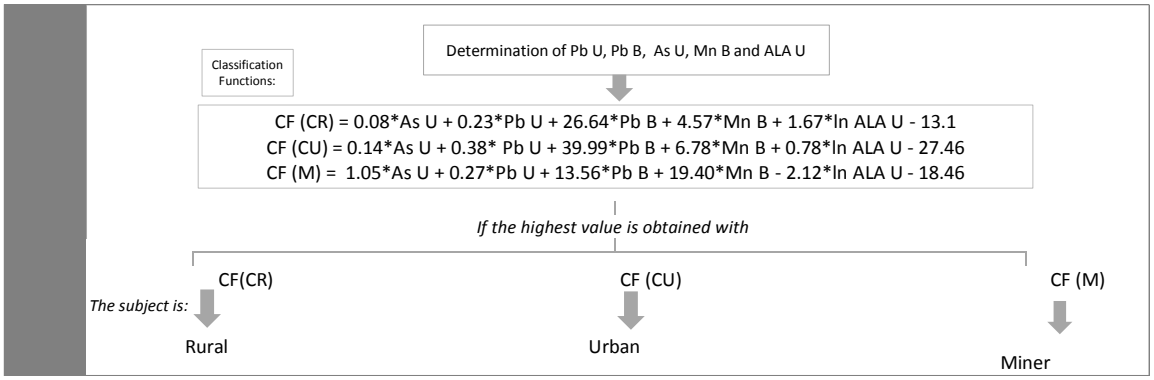


Fig. 6.7: Procedure I' proposed for individual assessment of the type of exposure in miners, urban and rural populations, through the determination of Pb and Mn in blood (B) and urinary (U) Pb, As and delta-ALA levels. Three classification functions (CF) were generated: for subjects non-occupationally exposed and living in a rural environment [CF (CR)] or in an urban environment [CF (CU)] and for miners [CF (M)]. The CF with the highest value indicates the type of exposure predicted by the model.

Assessment of the exposure in miners, urban and rural subjects (Procedure II')

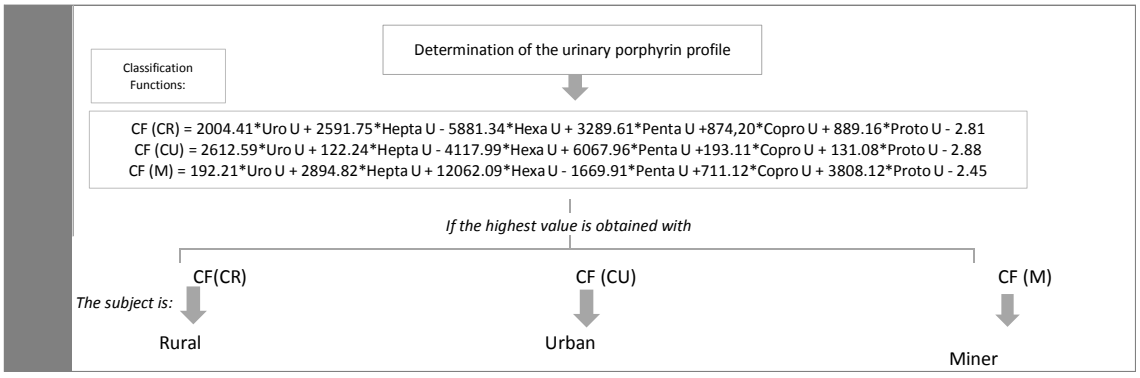


Fig. 6.8: Procedure II' proposed for individual assessment of the type of exposure in miners, urban and rural populations, through the determination of the urinary (U) porphyrins profile, the levels of Uro-, Hepta-, Hexa-, Penta-, Copro- and Protoporphyrins. Three classification functions (CF) were generated: for subjects non- occupationally exposed and living in a rural environment [CF (CR)] or in urban environment [CF (CU)] and for miners [CF (M)]. The CF with the highest value indicates the type of exposure predicted by the model.

6.3.3.1. Evaluation of the procedures

Procedure I'

Table 6.1: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR), through the integration of their Pb and Mn levels in blood and Pb, As and delta-ALA concentrations in urine by discriminant analysis. The table represents the type of exposure predicted by the model and the real exposure (Group).

		Predicted type of exposure		
Group	N			
		CR	CU	M
CR	10	9	1	0
CU	12	0	12	0
M	13	0	0	13

97.1% of the cases correctly classified

Procedure I' was applied to access if the type of exposure of each subject could be correctly identified through the combination of Pb and Mn levels in blood and the concentrations of Pb, As and delta-ALA in urine. With exception of one person living in the rural area, which was wrongly classified as belonging to the urban environment group, all subjects were properly classified by the model, corresponding to 97.1% of the cases correctly identified (Table 6.1).

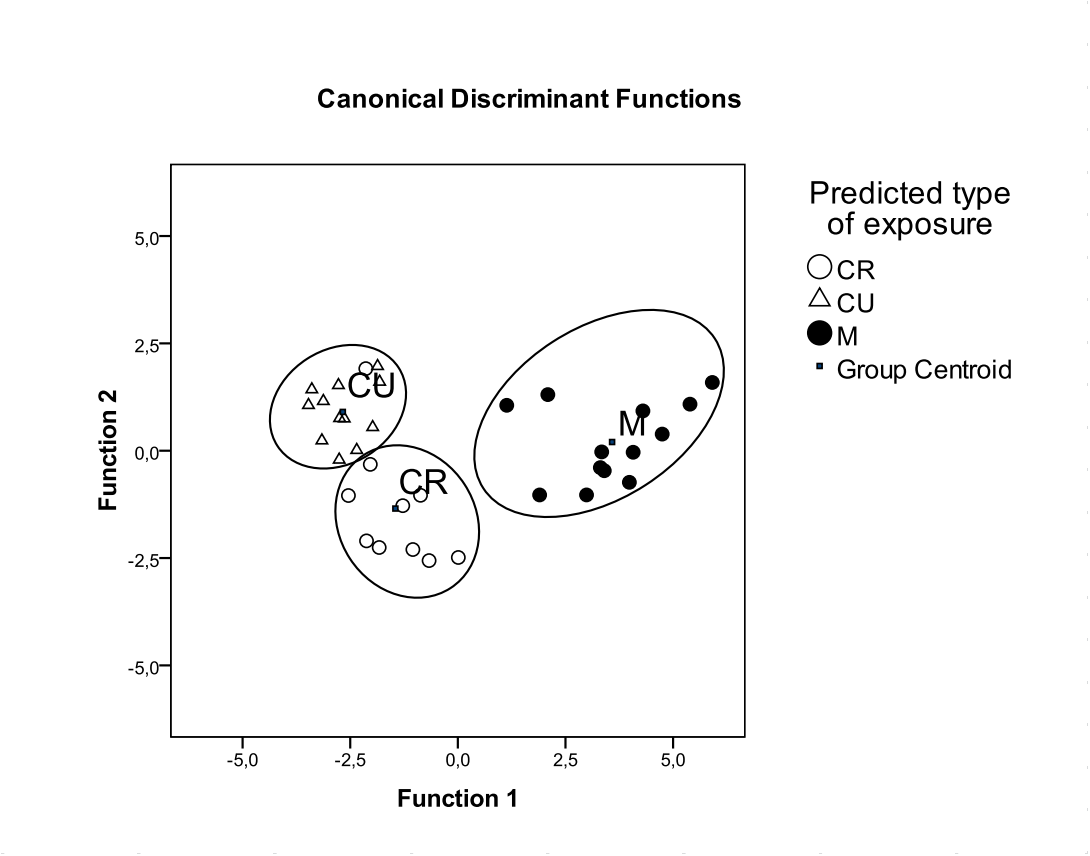


Fig. 6.9: Graphical representation of each subject classification according to the type of exposure, through the integration of its Pb and Mn levels in blood and urinary Pb, As and delta-ALA concentrations by discriminant analysis. A centroid value was calculated for each group and the results are plotted by the canonical discriminant functions.

The 3 groups, CR, CU and M, were clearly discriminated by Procedure I' showing clearly separated areas. However, one subject from the rural area was classified erroneously in the urban environment group, revealing to be distant from the area corresponding to its original population (Fig.6.9).

Procedure II'

Table 6.2: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR), using their individual urinary (U) porphyrin profile, the levels of uro-, hepta-, penta-, copro- and protoporphyrins. Discriminant analysis was performed. The table represents the type of exposure predicted by the model and the real exposure (Group).

		Predicted type of exposure		
Group	N	CR	CU	M
CR	20	10	6	4
CU	20	3	12	5
M	20	4	1	15

61.7% of the cases correctly classified

The assessment of the type of exposure in humans through the application of Procedure II' showed that the individual urinary porphyrin profile allowed that only 61.7% of the cases were correctly classified. Both rural and urban groups exhibited greater amount of errors as compared with the group of miners. In fact 75% of the miners were correctly identified while respectively 50% and 60%, of rural and urban subjects were classified appropriately by this procedure (Table 6.2).

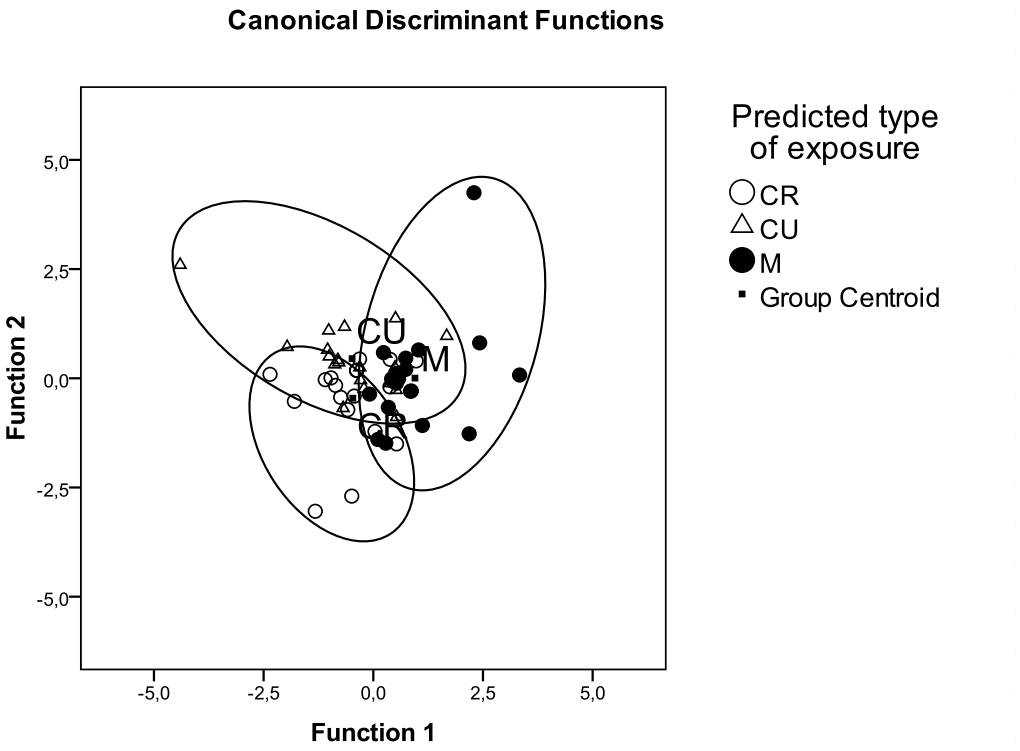


Fig. 6.10: Graphical representation of each subject classification according to the type of exposure, through its urinary porphyrin profile by discriminant analysis. A centroid value was calculated for each group and the results are plotted by the canonical discriminant functions.

Fig.6.10 shows that the areas of the 3 groups overlapped, revealing a poor discrimination capability of Procedure II'.

Testing Procedure I' through external data

Procedure I' was tested thereafter in 7 subjects who were randomly selected and were not included in the previous models. Using this procedure, six of the seven subjects were correctly classified according to the type of exposure (Table A5.1, appendix 5).

6.4. Discussion

Pb, As and Mn are neurotoxic metals co-occurring in several environmental settings, such as mines (Balbuena et al, 2011; Dahtrak and Nandi, 2009; Martinez-Finley et al., 2012; Yokel et al, 2009;) and since neurological disorders can clinically manifest only decades after the initial exposure (Gil and Pla, 2001), early predictive BMs have become increasingly important for the detection and diagnosis of the onset of these diseases (Kakkar and Jaffery, 2005; Scherer, 2005). Having some authors the opinion that exposed populations should be studied using a combination of exposure and/or effect BMs (Kakkar and Jaffery, 2005; Zhai et al, 2005), a multibiomarker approach was used to identify the exposure in a Portuguese population occupationally exposed to the mixture of Pb, As and Mn. Thus, in this work several BM of exposure and/or effect were applied to control the miners' exposure to this mixture.

6.4.1. Biomarkers of internal dose

Metal levels in blood and urine

Blood and urinary Pb concentrations of miners showed a trend to be higher than in non-occupationally exposed subjects. Higher standard deviations were also attained in this group, certainly explaining the lack of statistical differences (Figs 6.1a and 6.3a). Being this work a preliminary study to apply the results from the in vivo study (Chapters 4 and 5), further work with more subjects and the knowledge of other variables, such as the time of employment and confounding factors (e.g. smoking habits) would provide a clearer picture. In addition, NIOSH and ACGIH's BEIs are respectively 600 μg Pb/L and 300 μg Pb/L in blood [0.60 μg/g and 0.30 μg/g, considering a blood density of 1.0506 Kg/m^3 (Trudnowski and Rico, 1974)]. The mean Pb levels in blood observed in miners was 0.55 μg/g, with an upper limit of 0.81μg/g

suggesting that some workers might be at risk of excessive exposure to Pb. Despite their average urinary levels (30 μg/g creat) were within a range of urinary Pb concentrations described for urban populations (4-270 μg/g creat) (ASTDR, 2014), blood Pb levels are a more accepted BM (Moreira and Neves, 2008). Pb excretion in rural and urban populations was similar (Fig.6.3a), but urban subjects, with blood Pb levels within the range described in urban populations (ASTDR, 2014), had this parameter significantly ($p<0.05$) higher than in the rural group. The North of Lisbon is a very industrialized area and is also very populous, contributing for the degradation of the air quality. However, airborne-Pb measured over an urban-industrial corridor within metropolitan Lisbon in 2003 was sharply reduced as compared with the early 1990s. These findings were attributed to the widespread use of unleaded gasoline, introduced and fully enforced in 1990 and 1999 respectively (Freitas et al, 2003). The observed values are possibly attributed to the Pb disposition‘ characteristics, where the skeleton represents the largest and kinetically slow pool (having a half-life of more than 20 years), with Pb in bone may contributing as much as 50% to Pb in blood (Casarett and Doull’s, 2007).

Figs. 6.1b and c and 6.3b and c shows that there was a trend for the miners to have increased levels of As and Mn in both peripheral samples, comparing to the other two groups. This difference was more marked in the urine, were a statistical significance ($p<0.05$) was observed. Concerning to As, the result is in agreement with the information that urinary As has been the most used BM to indicate As exposure (Marchiset-Ferlay et al, 2012). The mean values observed in the miners group seems to indicate an excessive exposure to As, when comparing their As levels in blood (0.003 μg/g) with references of 0.5- 2 μg/L in non exposed subjects and 2-41 μg/L upon exposure to contaminated drinking water (ASTDR, 2007a, MAK, 2002). The comparison of their urinary As mean value (15.31 μg/g creat) with a study with copper smelters, which revealed average values of 16.9 μg/g (range 2.6 to 50.8 μg/g creat) (ASTDR, 2007a) supports this awareness. Blood and urinary levels of As in urban subjects (respectively, 0.0020 μg/g and 0.78 μg/g creat), which actually were similar to the rural

population (0.0022 μg/g in blood and 1.39 μg/g creat in urine (Fig. 6.1b and 6.3b), were not suggestive of an excessive exposure.

While no reliable BMs are established for Mn, in this work urinary determinations seemed to indicate better the exposure than blood values do, disagreeing with several other studies (ASTDR, 2007c) possibly duo to the small size of our sample. Even so, when comparing Mn proposed BEI values in blood of <15 μg/L (MAK Commission) and <10 μg/L (Lauwerys and Hoet, 2011) and the description of 4-15 μg/L in the blood of non exposed subjects (Santamaria, 2008), the miners (exhibiting 0.013 μg/g Mn blood values) are possibly excessively exposed to Mn. In the same way, the limit exposure levels proposed by Lauwerys and Hoet, 2011 in urine (<3μg/g creat) are lower than the values observed in this group of workers (10.86 μg/g creat). The urban subjects presented inconsistent results pertaining to the levels of Mn in peripheral samples. In fact while in blood there was only a slight and not statistical significant increase when compared with the rural group (Fig. 6.1c), the urinary excretion was significantly lower than in rural subjects (Fig.6.3c), reflecting again the need of suitable BMs to indicate Mn exposures. Even so, the obtained results did not indicate excessive exposure (Fig. 6.1c and 6.3c).

At a general rule, the results obtained with the human populations are in agreement with the experimental results, since increased levels of the three metals in mixture treated rats/exposed human group, as compared with controls, were observed in both cases (Fig. 3.2 and 3.6, Chapter 3; Fig.6.1a, b and c and Fig.6.3a, b and c). However limitations exist, such as the impossibility of comparing single and co-exposed humans and ascertain if in the co-exposed population there was a higher deposition of Pb in the brain and/or increased neurotoxicity, then in the control groups.

6.4.2. Biochemical markers

Neurochemical and heme synthesis endpoints

AChE activity in the blood of miners was significantly ($p<0.05$) higher than in the rural population (Fig.6.2), in accordance with the trend observed in experimental data where mixture treated rat's AChE activity was higher than controls, despite the alteration lacked statistical significance (Fig. 4.5, Chapter 4). This parameter can show a biphasic response (Chapter 4), corresponding the first phase to its increase due to the low levels of metals exposure, in the same way as is suggested in the miners (Fig.6.1a, b and c and 6.3a,b and c). The reason why the urban population had the highest ($p<0.05$) AChE activity in blood demands further explanation (Fig.6.2).

Differently from experimental observations (Fig. 4.7), delta-ALA levels in the urine of miners exhibited only a very slight and not significant increase, as compared with the other two groups of subjects (Fig. 6.4). High standard deviations were observed, possibly due to polymorphisms in genes for the enzyme ALAD (Bergdahl et al., 1997) and also due to the small number of subjects. In addition, delta-ALA levels of 3 mg/g creat are proposed as a threshold to detect levels of Pb in blood equal to or higher than 20 µg/dL (Caldeira et al, 2000), for which adverse health effects including neurological alterations are described (ASTDRb, 2007), suggesting that the finding of 4.36 mg/g creat as the highest delta-ALA levels in the miners justifies further studies.

Total porphyrins in the urine of miners and urban subjects were significantly ($p<0.05$) higher than in the rural population (Fig.6.5) and similarly, Pb/As/Mn mixture –treated rats increased the excretion of these heme precursors as compared with the controls (Fig.4.8, Chapter 4). Woods et al. (2009) described urinary total porphyrins of 35.2µg/g creat in normal subjects, while miners and urban subjects presented mean values of 18.70 µg/g creat and 17.45 µg/g creat, respectively, which do not seem to indicate cause of concern respecting to porphyrins accumulation.

After analyzing the urinary porphyrin profile (Fig.6.6), the results obtained in humans seem to be even more inconsistent then the ones observed in the rats (Fig.4.9, Chapter 4), at least when considering each porphyrin alone. Currently, the human populations are exposed to mixtures of these 3 metals, certainly including more metals, which might also interfere with heme biosynthesis (Pingree et al, 2001). Since different metals can have different effects on porphyrins, the complexity of eventual additional interactions might explain some of the inconsistency of the results. When considering each porphyrin alone, the confrontation of the obtained values (Fig. 6.6) with the urinary porphyrin profile of normal subjects, with 20.7 nmol/g creat of uroporphyrins, 4.1 nmol/g creat of heptaporphyrins, 0.89 nmol/g creat of hexaporphyrins, 4.45 nmol/g creat of pentaporphyrins and 195 nmol/g creat of coproporphyrins (Kern et al, 2011), does not suggest relevant alterations in any of the studied groups.

Each BM alone was tested with respect to its capability to predict the type of exposure (Tables A4.1-11, appendix 4) and revealed a weaker prediction capability as compared with their combination, as will be testified.

6.4.3. Combination of biomarkers

The phase 1 of the procedures I and II (Figs.5.2 and 5.3, Chapter 5) was translated to humans as Procedures I' and II' (Fig.6.7 and 6.8). Our goal was to use the same combination of BMs that was used in the rats (Chapter 4), to identify whether each subject was living in a rural environment, was exposed to metals in an urban setting or was occupationally exposed in mines. Moreover, we intended to investigate if the procedures could be applied to distinguish persons living in an urban and rural environment, as urban environment may be polluted with traces of these 3 metals.

Procedure I', allowed to correctly classify 97.1% of the cases, with only 1 of 35 subjects erroneously identified as living in a urban environment, when in fact it was a person from the rural group (Table 6.1). This person was actually quite distant from its original group (Fig.6.9). Due to the fact that this is a preliminarily study no data is available concerning the history of the subject and thus, it is not possible to conclude if a procedure failure occurred while classifying the subject. Differently all miners were correctly identified (Table 6.1), with a clear separation from the other two groups (Fig.6.9).

Procedure II' was also evaluated as a more practical and advantageous alternative, where using the urinary porphyrins profile could only imply one analytical determination. However, and differently from what was experimentally observed (Table 5.4, Chapter 5), only 61.7% of the subjects were correctly classified (Table 6.2) with Fig.6.10 showing that this procedure had a poor separation capability among groups. Despite the miners 'group did not detached from the other two groups (Fig. 6.10), 75% of miners were correctly identified (Table 6.2), which is a motive to confirm these studies using a higher number of samples. In fact and considering the instability of porphyrins in the samples that were transported from Alentejo to Lisbon, further studies are necessary to clarify if the transport procedure was inadequate.

After constructing prediction models an immediate question may arise, "what will be its applicability to other exposed persons in similar contexts?" Hence, procedure I' was later applied in 7 randomly selected individuals, which data were not used to construct the models. It must be highlighted that using 7 persons is largely far from being sufficient and that no valid conclusion can be extracted from such a small preliminary test. Even so, only one of the 7 cases was erroneously identified (Table A5.1, appendix 5) leading the will of further robust confirmation.

6.5. Conclusions

This work suggests that some workers from the studied population of miners might be exposed to excessive levels of Pb, As and Mn. When considering the co-occurrence of these metals in their workplace, we suggest that in particular the exposure to Pb should be controlled. The translation of the results obtained from experimental data revealed that the use of the selected BMs, Pb and Mn blood levels and urinary Pb, As and delta-ALA levels to identify exposed workers and identify urban environmental exposures was promising when the parameters were applied in combination. We believe that this approach may possibly contribute to prevent the risk of neurotoxicity of these exposed populations.

Chapter 7

Final discussion and conclusions

7.1. Final discussion

Pb, As and Mn are among the major toxic contaminants in mining environments (Dahtrak and Nandi, 2009). Considering that the three metals have neurotoxic effects (Finkelstein et al, 1998; Rodriguez et al, 2003; Wong, 2006) it was hypothesized that mine workers could be at risk of increased neurotoxicity. In Chapters 3 and 4 an in vivo assay was performed with rats treated with each single metal, Pb, As and Mn and co-treated with the mixture. The aim was to investigate the mechanism of interaction among these 3 metals in order to select endpoints of toxicity to apply as BMs to control and prevent the risk of exposure to this mixture. To achieve this objective we evaluated the changes in: 1) behavioral motor activities; 2) metals' levels in target organs, blood and urine; 3) and biochemical markers in brain, blood and urine. The results evidence that upon the co-exposure to Pb, As and Mn behavioral toxicity was higher than upon single exposures (Chapter 4).

In Chapter 3, the interactions among the 3 metals were investigated, and it was observed a higher deposition of Pb in rat kidney and brain, as compared with metal deposition after treatments with each single metal. However, blood Pb levels did not reflect brain status, concerning to the increased Pb accumulation; it raises concern to an underestimation of the risk of neurotoxicity in humans, since the exposure to this metal is frequently biomonitored through blood Pb measurements (ASTDRc, 2007).

Subsequently, in Chapter 4, brain Pb levels were correlated with behavioral changes suggesting a causal relation between Pb accumulation in brain and motor activity dysfunctions. Neurochemical endpoints were also determined and decreased activity of AChE in the brain and increased delta-ALA levels were found. The increase of delta-ALA in rats co-exposed to the mixture, may contribute to increase of motor dysfunction in the same group of rats as it is documented that in excess delta ALA can act as a neurotoxin (Ennis et al., 2003).

Considering that neurotoxicity induced by chronic metal exposure can be progressive and may clinically manifest only decades after the initial exposure (Gil and Pla, 2001) our goal was to select early BMs to be applied in the control of chronic occupational exposure to the mixture of Pb, As and Mn. The results of the in vivo assay showed that when comparing controls and/or single exposed groups, in rats treated with the metal mixture, there was an increase of blood or urinary metal levels, augments in the concentrations of serum PRL, urinary delta-ALA, total porphryrins and changes in the urinary porphyrin profile. The correlations of these markers with brain and/or behavioral parameters (Chapters 4 and 5), suggested their suitability as peripheral BMs of exposure and/or neurotoxicity upon exposure to the mixture of Pb, As and Mn (Chapter 4). However when used alone, none of the BMs revealed to be accurate enough and could be applied only to one type of exposure (Chapter 4 and 5). This was in fact an expected outcome due to the complexity of the nervous system and to the multi-mechanisms of toxicity of the different metals (Quinones and Daouk, 2009; Rachakonda et al, 2004).

In chapter 5 a multibiomarker procedure was proposed based on a novel approach of BMs´ integration methodologies, which was already employed to diagnose disorders with multifactorial etiologies, such as cancer and PD or AD (Ahmed et al, 2010; Da et al, 2014; Edgell et al, 2010). In that sense, the determined BMs were selected and combined aiming to provide a tool that could timely identify exposed rats and semi-quantitatively predict the magnitude of neurotoxic effects induced by the co-exposure to the mixture of Pb, As and Mn, with the purpose of a further translation to humans' studies. Owing to each personal time of exposure, life habits, and different individual susceptibility to metal exposures, there is a need to develop an adequate tool to be applied to each subject, rather than merely in a group basis. In this perspective two procedures were generated: one combining the levels of Pb, Mn and AChE activity in blood, serum PRL and urinary Pb, As, delta-ALA and total porphyrins, while the second procedure integrated the urinary porphyrin profile, as a more practical alternative. Both procedures revealed to be suitable to achieve the proposed objectives better

than the traditional approach, focused on the search of a strong BM to be used alone (Chapter 5).

In chapter 6, the collection of preliminary data pertaining to levels of exposure to metals in Portuguese mines leaded to find that the studied population of workers might be exposed to excessive levels of Pb, As and Mn (Chapter 6), as compared with reference limit values as well as after comparing with results from other studies (ASTDRa, 2007; ASTDR, 2014; Lauwerys and Hoet, 2011; MAK, 2002; Santamaria, 2008). Given the co-occurrence of these metals in their workplace, exposure levels to Pb should be investigated with more detail. The pertinence of exploring BMs' integration methodologies to detect exposed workers while in a still potentially reversible stage was suggested from the experimental results; thus, these results were tested in a risky population of Portuguese miners co-exposed to Pb, As and Mn. The combination of the several BMs in blood and urine of the exposed population seemed encouraging concerning the identification of the exposure, differently from the integration of the urinary porphyrin profile, justifying further studies with more samples.

Some authors consider a naïve expectation that a single BM can capture the intricate process underlying a CNS illness (Quinones and Daouk, 2009), but it is also unreal to expect that the predictive procedure developed in this work can function as "a magical formula". Rather, this work intended to test BMs' integration methodologies in the context of occupational exposure to a mixture of these metals. It is plausible that the integration of different BMs, such as susceptibility BMs, could also be considered and might have an even better applicability. A relevant outcome is the suggestion that weak BMs can gain predictive strength when used together. In fact, while no better BMs are found concerning some type of exposures, the combination of the available weaker ones can be considered a good tool to use and explore.

However, and as in many science issues, the rapprochement to some answers upsurges an even greater number of questions. The tested procedures were generated from toxicity data with the same dose, being important to understand if the procedure would maintain its suitability with different doses. Moreover, while in human scenarios accidental single exposures generally occurs to high chemical's levels and co-exposures frequently occur at chronic low levels, the applied procedures were generated from data obtained using low doses. Biphasic responses are also described for some of the combined BMs (de Lima, 2013; Herrera et al, 2013) and also for the effects (Chandra et al., 1981; Reddy et al., 2003; Rocha et al., 2001; Rodrigues et al., 1996), being yet to be known how would the models respond to the use of threshold doses. In addition, miners are co-exposed to other metals beyond Pb, As and Mn, which may interfere with the integrated BMs being important to ascertain their potential influence in these models.

Finally, in real life scenarios people are co-exposed to an almost infinite number of chemical mixtures and thus, it is barely impossible to determine all the components of a mixture present in an environmental setting (ASTRD, 2004). For all practical purposes, if mixtures´ components are not usually well known, criteria are needed to define the relevant components of a mixture. Such criteria cannot rely simply on the concentrations of the compounds in the mixture, but must also take note of the expected contribution to relevant endpoints of toxicity (Kortenkamp and Faust, 2009). Recently, it has been argued that grouping criteria should focus on common adverse outcomes (Kortenkamp and Faust, 2009), leading to wonder if integrating specific but also some unspecific BMs could lead to better results, covering common effects of more chemicals. It is a possibility that the integration of BMs relied on previous identification of key components of a mixture from specific environmental settings may be a valid approach to monitor persons exposed to complex mixtures of chemicals.

7.2. Final conclusions

This work established that:

1- In the experimental co-exposure of rats to Pb, As and Mn, it was observed:

a) An increase in motor activity impairment compared to single exposures;

b) An augment of Pb's deposition in kidney and brain as compared with rats treated with Pb alone, while blood Pb levels fail to reflect these alterations;

c) An induction of neurochemical modifications in the brain, such as decreased AChE activity and accumulation of delta-ALA (which can act as a neurotoxin), more than upon single exposures;

d) A change in peripheral parameters, such as blood and urinary metal levels, serum PRL, urinary delta-ALA, total porphryrins and the urinary porphyrin profile;

2- The multivariate methodology used to integrate BMs is an useful tool for prediction of the type of exposure and the magnitude of the induced neurotoxic effects upon co-exposure to Pb, As and Mn

3- The application of a biomarker approach in a population of miners to control their exposure to Pb, As and Mn suggested that the integration of Pb and Mn blood levels and urinary Pb, As and delta-ALA levels could be used to identify exposed workers, and may represent a tool to prevent the risk of neurotoxicity in these occupational cohorts.

7.3. Future perspectives

Our future work will be focused in a new toxicological in vivo model and in the implementation of the risk assessment in miners exposed to the studied metals mixture.

Thus, we intend:

1- To perform an in vivo assay with rats exposed to the same mixture of Pb, As and Mn increasing Pb doses, for a longer exposure period and using other behavioral evaluations, determining the same studied BMs and exploring the applicability of integrating BMs of susceptibility.

2- To continue and optimize the research in the same mining company after screening the metals content in mine airborne, increasing the number of persons under study and collecting personal details concerning these subjects. The workers will be grouped according to high, medium and low level exposures with the aim of improving the predictive procedures.

Blood and urine samples will be collected for determination of Pb, As and Mn as well as other metals which could interact with them. All the other biochemical BMs will be determined in the same samples.

Neurobehavioral and performance tests will be implemented in collaboration with a clinical psychologist (including the control population) to identify subtle changes in motor and memory dysfunction. The neuropsychological test battery includes tests of motor function, response speed, memory, attention and executive functions.

3- Finally, the multiparameter model with procedures I and II will be tested in this risky population in order to select the combination of BMs to apply in risk assessment to mixtures of these neurotoxic metals.

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Appendices

Appendix 1

Table A1.1: Identification of controls (C) and Pb treated rats through its levels of Pb in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus their real exposure (Group).

Group	N	Predicted type of exposure	
		C	Pb
C	6	2	4
Pb	6	2	4

50.0% of the cases correctly classified

Table A1.2: Identification of controls (C) and Pb treated rats through its levels of Pb in blood, bydiscriminant analysis. The table represents the type of exposure predicted by the model versus their real exposure (Group).

Group	N	Predicted type of exposure	
		C	Pb
C	6	5	1
Pb	6	2	4

75.0% of the cases correctly classified

Table A1.3: Identification of controls (C) and As exposed rats through its levels of As in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus their real exposure (Group).

Group	N	Predicted type of exposure	
		C	As
C	6	6	0
As	6	2	4

83.3% of the cases correctly classified

Table A1.4: Identification of controls (C) and As exposed rats through its levels of As in blood, by discriminant analysis. The table represents the type of exposure predicted by the model versus their real exposure (Group).

Group	N	Predicted type of exposure	
		C	As
C	6	4	2
As	6	4	2

50.0% of the cases correctly classified

Table A1.5: Identification of controls (C) and Mn exposed rats through its levels of Mn in urine by discriminant analysis. The table represents the type of exposure predicted by the model versus their real exposure (Group).

Group	N	Predicted type of exposure	
		C	Mn
C	6	2	4
Mn	6	0	6

66.7% of the cases correctly classified

Table A1.6: Identification of controls (C) and Mn exposed rats through its levels of Mn in blood by discriminant analysis. The table represents the type of exposure predicted by the model versus their real exposure (Group).

Group	N	Predicted type of exposure	
		C	Mn
C	6	6	0
Mn	6	0	6

100.0% of the cases correctly classified

Table A1.7: Identification of the exposure, to Pb, As, Mn or the mixture of Pb/As/Mn of each rat through its levels of delta-ALA in urine, by discriminant analysis. C represents control rats. The table shows the type of exposure predicted by the model versus their real exposure (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	4	1	1	0	0
As	6	1	4	1	0	0
Mn	6	1	3	2	0	0
Pb	6	0	1	0	5	0
Mixt	6	0	0	0	2	4

63.3% of the cases correctly classified

Table A1.8: Identification of the exposure, to Pb, As, Mn or the mixture of Pb/As/Mn of each rat through its levels of total porphyrins in urine, by discriminant analysis. C represents control rats. The table shows the type of exposure predicted by the model versus their real exposure (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	6	0	0	0	0
As	6	0	3	1	2	0
Mn	6	2	0	4	0	0
Pb	6	0	2	0	4	0
Mixt	6	0	4	0	0	2

63.3% of the cases correctly classified

Table A1.9: Identification of the exposure, to Pb, As, Mn or the mixture of Pb/As/Mn of each rat through its levels of serum prolactin, by discriminant analysis. C represents control rats. The table shows the type of exposure predicted by the model versus their real exposure (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	3	0	0	3	0
As	6	0	4	1	1	0
Mn	6	0	1	2	2	1
Pb	6	2	0	3	3	1
Mixt	6	1	3	0	0	1

43.3% of the cases correctly classified

Table A1.10: Identification of the exposure, to Pb, As, Mn or the mixture of Pb/As/Mn of each rat through its levels of urinary (U) uroporphyrins, by discriminant analysis. C represents control rats. The shows the type of exposure predicted by the model versus their real exposure (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	6	0	0	0	0
As	6	1	2	1	1	1
Mn	6	0	2	2	1	1
Pb	6	0	1	1	1	3
Mixt	6	0	0	1	1	4

50.0% of the cases correctly classified

Table A1.11: Identification of the exposure, to Pb, As, Mn or the mixture of Pb/As/Mn of each rat through its levels of urinary (U) heptaporphyrins, by discriminant analysis. C represents control rats. The table shows the type of exposure predicted by the model versus its real exposure (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	5	0	1	0	0
As	6	1	1	2	2	0
Mn	6	2	1	2	0	1
Pb	6	1	3	0	1	1
Mixt	6	0	1	1	2	2

36.7% of the cases correctly classified

Table A1.12: Identification of the exposure, to Pb, As, Mn or the mixture of Pb/As/Mn of each rat through its levels of urinary (U) hexaporphyrins, by discriminant analysis. C represents control rats. The table shows the type of exposure predicted by the model versus their real exposure (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	3	0	2	1	0
As	6	0	1	1	3	1
Mn	6	2	0	3	1	0
Pb	6	0	2	2	2	0
Mixt	6	0	2	0	0	4

43.3% of the cases correctly classified

Table A1.13: Identification of the exposure, to Pb, As, Mn or the mixture of Pb/As/Mn of each rat through its levels of urinary (U) pentaporphyrins, by discriminant analysis. C represents control rats. The table shows the type of exposure predicted by the model versus their real exposure (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	4	0	2	0	0
As	6	1	4	1	0	0
Mn	6	2	4	0	0	0
Pb	6	1	0	0	5	0
Mixt	6	0	0	0	0	6

63.3% of the cases correctly classified

Table A1.14: Identification of the exposure, to Pb, As, Mn or the mixture of Pb/As/Mn of each rat through its levels of urinary (U) coproporphyrins, by discriminant analysis. C represents control rats. The table shows the type of exposure predicted by the model versus their real exposure (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	3	1	2	0	0
As	6	2	3	0	1	0
Mn	6	1	0	5	0	0
Pb	6	1	2	0	2	1
Mixt	6	0	0	0	3	3

53.3% of the cases correctly classified

Table A1.15: Identification of the exposure, to Pb, As, Mn or the mixture of Pb/As/Mn of each rat through its levels of urinary (U) protoporphyrins, by discriminant analysis. C represents control rats. The table shows the type of exposure predicted by the model versus their real exposure (Group).

		Predicted type of exposure				
Group	N	C	As	Mn	Pb	Mixt
C	6	1	0	2	2	1
As	6	0	2	2	0	2
Mn	6	0	1	2	2	1
Pb	6	0	0	1	5	0
Mixt	6	0	0	1	2	3

43.3% of the cases correctly classified

Appendix 2

Table A2.1: Relationship of each BM, urinary (U) Pb, As, Mn , delta aminolivulinic acid (ALA) and total porphyrins (Port T), levels of Pb, As and Mn in blood (B) and urine (U) and serum (S) prolactin (PRL S), with motor activity, ambulation and rearing counts, in rats exposed to Pb, As, Mn or to the mixture of Pb/As/Mn. For significant ($p<0.05$) regressions the goodness-of-fit of linear regression is represented (R^2). "NS" indicates not significant regressions.

		Group			
		Pb	As	Mn	Mixt
Pb U	Ambulation	NS	-	-	-
	Rearing	NS	-	-	-
Pb B	Ambulation	R^2=0.389	-	-	-
	Rearing	NS	-	-	-
As U	Ambulation	-	R^2=0.718	-	-
	Rearing	-	R^2=0.628	-	-
As B	Ambulation	-	R^2=0.610	-	-
	Rearing	-	R^2=0.486	-	-
Mn U	Ambulation	-	-	NS	-
	Rearing	-	-	NS	-
Mn B	Ambulation	-	-	R^2=0.370	-
	Rearing	-	-	R^2=0.500	-
ALA U	Ambulation	NS	NS	NS	R^2=0.877
	Rearing	NS	NS	NS	R^2=0.739
Porf T U	Ambulation	NS	R^2=0.519	NS	R^2=0.865
	Rearing	NS	R^2=0.499	NS	R^2=0.860
Uro U	Ambulation	NS	R^2=0.641	R^2=0.490	R^2=0.679
	Rearing	NS	R^2=0.803	R^2=0.686	R^2=0.528
Hepta U	Ambulation	R^2=0.625	NS	NS	R^2=0.812
	Rearing	NS	NS	NS	R^2=0.630
Hexa U	Ambulation	NS	NS	NS	R^2=0.780
	Rearing	NS	NS	NS	R^2=0.697
Penta U	Ambulation	R^2=0.591	NS	NS	R^2=0.901
	Rearing	NS	NS	NS	R^2=0.818
Copro U	Ambulation	NS	NS	R^2=0.373	R^2=0.756
	Rearing	NS	NS	R^2=0.520	R^2=0.747
Proto U	Ambulation	NS	NS	NS	NS
	Rearing	NS	NS	NS	NS
PRL S	Ambulation	NS	R^2=0.564	NS	R^2=0.334
	Rearing	NS	R^2=0.419	NS	NS

The equations of significant regressions ($p<0.05$) which were exclusive of only one group were used to estimate the motor activity of each rat, as is described in the following tables.

Table A2.2: Estimation of ambulation counts of rats exposed to Pb through its levels of urinary (U) Pb, by linear regression. The values predicted by the model are compared with the values observed in each rat.

Individual (code number)	ln Pb U (µg/L)	Motor activity Ambulation counts		
		Predicted	Observed	Error
25	-3,22	43	22	21
26	-2,12	37	18	19
27	-2,66	40	9	31
28	0,03	26	21	5
29	-1,97	36	29	7
30	-0,4	28	18	10
			averge error:	15 ± 10

Ambulation counts = -5.21 ln Pb U + 25.92

Table A2.3: Estimation of motor activity, ambulation and rearing counts, of rats exposed to As through its levels of urinary (U) As, by linear regression. The values predicted by the model are compared with the values observed in each rat.

Individual (code number)	ln As U (µg/L)	Motor activity					
		Ambulation counts			Rearing counts		
		Predicted	Observed	Error	Predicted	Observed	Error
13	-0,04	10	8	2	17	14	3
14	0,18	9	12	3	16	19	3
15	-1,24	16	20	4	21	28	7
16	-0,42	12	20	8	18	23	5
17	-0,53	12	2	10	19	14	5
18	-1,35	16	12	4	21	13	8
			averge error:	5 ± 3		averge error:	5 ± 2

Ambulation counts = -4.94 ln As U + 9.65

Rearing counts = -3.52 ln As U + 16.66

Table A2.4: Estimation of motor activity, ambulation and rearing counts, of rats exposed to As through its levels of As in blood (B), by linear regression. The values predicted by the model are compared with the values observed in each rat.

Individual (code number)	ln As B (µg/L)	Motor activity					
		Ambulation counts			Rearing counts		
		Predicted	Observed	Error	Predicted	Observed	Error
13	-0,04	10	8	2	17	14	3
14	-1,56	17	12	5	22	19	3
15	0,01	10	20	10	17	28	11
16	-0,65	13	20	7	19	23	4
17	-0,66	13	2	11	19	14	5
18	-1,42	16	12	4	22	13	9
			averge error:	7 ± 3		averge error:	6 ± 3

Ambulation counts = -4.76 ln As B + 9.66

Rearing counts = -3.24 ln As B + 16.99

Table A2.5: Estimation of motor activity, ambulation and rearing counts, of rats exposed to Mn through its levels of Mn in blood (B), by linear regression. The values predicted by the model are compared with the values observed in each rat.

Individual (code number)	ln Mn B (µg/L)	Motor activity					
		Ambulation counts			Rearing counts		
		Predicted	Observed	Error	Predicted	Observed	Error
13	-1,02	17	22	5	16	14	7
14	-1,32	19	15	4	18	19	1
15	-0,87	16	30	14	15	28	12
16	-1,2	18	14	4	17	23	6
17	-1,21	18	8	10	17	14	3
18	-1,08	17	6	11	17	13	4
			averge error:	8 ± 4		averge error:	5 ± 4

Ambulation counts = -7.03 ln Mn B + 9.76

Rearing counts = -6.32 ln Mn B + 9.76

Table A2.6: Estimation of motor activity, ambulation and rearing counts, of rats exposed to the mixture of Pb/As/Mn through its levels of urinary (U) hexaporphyrins (Hexa), by linear regression. The values predicted by the model are compared with the values observed in each rat.

Individual (code number)	ln Hexa U nmol/g creat)	Motor activity					
		Ambulation counts			Rearing counts		
		Predicted	Observed	Error	Predicted	Observed	Error
7	-5,55	0	1	1	3	0	3
8	-6,57	9	5	4	12	12	0
9	-6,32	6	2	4	10	5	5
10	-6,81	11	4	4	14	12	2
11	-6,81	11	4	7	14	5	9
12	-6,57	9	7	2	12	7	5
			averge error:	4 ± 4		averge error:	4 ± 3

Ambulation counts = -9.9 ln Hexa U - 56.35

Rearing counts = -9.2 ln Hexa U - 48.23

Appendix 3

Table A3.1: Prediction of motor activity, ambulation and rearing counts of rats exposed to Pb, As, Mn or the mixture of the three elements through the integration of BMs, the levels of Pb, Mn and AChE activity in blood (B), PRL in serum (S) and urinary (U) levels of Pb, As, ALA and total porphyrins. Data were analyzed by multiple linear regression. All the values estimated by the model are compared with the number of movements observed in each rat.

		BMs Levels								Motor activity					
										Ambulation counts			Rearing counts		
Rat (code number)	Group	ln As U (µg/ g creat)	ln Pb U (µg/ g creat)	ln Mn B (µg/ g blood)	ln Pb B (µg/ g blood)	ln AChE B (nmol/min/g blood)	ln PRL S (ng/ mL serum)	ln ALA U (mg/g creat)	ln Porf T U (µg/ g creat)	Predicted	Observed	Abs Error	Predicted	Observed	Abs Error
13	As	-0,04					3,55		-6,44	12	8	4	18	14	4
14	As	0,18					3,79		-6,44	9	12	3	16	19	3
15	As	-1,24					3,92		-6,81	15	20	5	20	28	8
16	As	-0,42					3,41		-6,57	15	20	5	19	23	4
17	As	-0,53					3,94		-7,42	8	2	6	17	14	3
18	As	-1,35					3,98		-6,91	15	12	3	21	13	8
											Average error:	4 ± 1		Average error:	5 ± 2
19	Mn			1,47			3,72		-7,42		22		17	14	3
20	Mn			1,29			3,92		-8,11		15		14	19	5
21	Mn			1,13			3,51		-7,42		30		19	28	9
22	Mn			1,15			3,43		-7,6		14		18	23	5
23	Mn			1,04			3,36		-8,11		8		16	14	2
24	Mn			1,16			3,53		-7,67		6		17	13	4
														Average error:	5 ± 3
25	Pb		-3,22		1,49	-1,14	3,68	-4,25	-7,01	25	22	3	28	24	4
26	Pb		-2,12		1,44	-1,08	3,36	-3,76	-6,32	18	18	0	21	23	2
27	Pb		-2,66		1,25	-2,14	2,74	-4,64	-7,01	11	9	2	6	5	1
28	Pb		0,03		1,43	-0,29	3,30	-4,14	-6,5	22	21	1	31	31	0
29	Pb		-1,97		1,72	-1,94	3,20	-4,17	-6,81	22	29	7	22	27	5
30	Pb		-0,4		1,24	-1,91	3,30	-4,06	-6,81	21	18	3	25	21	4
											Average error:	3 ± 3		Average error:	3 ± 2
7	Mixt	-0,39		-0,77	1,27				-4,82	2	1	1	1	0	1
8	Mixt	0,18		-1,05	1,28				-5,99	3	5	2	8	12	4
9	Mixt	0,32		-1,16	1,42				-4,44	0	2	2	5	5	0
10	Mixt	-1,97		-0,87	1,42				-6,38	13	4	9	14	12	2
11	Mixt	-0,84		-0,92	1,18				-6,32	8	4	4	9	5	4
12	Mixt	-1,66		-1,07	1,86				-6,65	12	7	5	20	7	13
											Average error:	4 ± 3		Average error:	4 ± 4

Table A3.2: Prediction of motor activity, ambulation and rearing counts, of rats exposed to Pb, As, Mn or the mixture of the three elements through its urinary (U) porphyrins, uro-, hepta, hexa-, penta-, copro- and protoporphyrins. Data were analyzed by multiple linear regression. The values estimated by the model were compared with the number of movements observed in each rat.

BMs Levels							Motor activity					
							Ambulation counts			Rearing counts		
Rat (code number)	Group	ln Uro U	ln Hepta U	ln Penta U (nmol/ g creat)	ln Copro U	ln Proto T U	Predicted	Observed	Abs Error	Predicted	Observed	Abs Error
13	As	-6,65	-6,5	-7,13	-3,13		9	8	1	18	14	4
14	As	-6,57	-6,44	-7,13	-2,93		13	12	1	21	19	2
15	As	-6,91	-6,73	-7,42	-2,75		14	20	6	20	28	8
16	As	-5,52	-5,07	-7,26	-2,34		20	20	0	23	23	0
17	As	-7,13	-6,65	-7,26	-3,09		6	2	4	15	14	1
18	As	-7,82	-8,52	-9,21	-2,74		12	12	0	14	13	1
								Average error:	2 ± 3		Average error:	3 ± 3
19	Mn	-6,81	-9,21		-3,52	-7		22		4	14	10
20	Mn	-6,57	-6,32		-3,77	-7,4		15		4	19	15
21	Mn	-6,5	-6,07		-3,22	-7,1		30		7	28	21
22	Mn	-7,42	-8,11		-5,91	-8,1		14		0	23	23
23	Mn	-7,01	-7,01		-4,32	-7,8		8		0	14	14
24	Mn	-6,78	-6,75		-3,77	-7,4		6		3	13	10
											Average error:	16 ± 5
25	Pb	-8,11	-6,38			-9,2	19	22	3		24	
26	Pb	-7,01	-6,12			-7,8	19	18	1		23	
27	Pb	-6,73	-6,21			-9,2	15	9	6		5	
28	Pb	-7,42	-6,27			-9,2	17	21	4		31	
29	Pb	-7,26	-7,82			-8,1	30	29	1		27	
30	Pb	-6,44	-6,21			-7,4	20	18	2		21	
								Average error:	3 ± 2			
7	Mixt	-6,81		-4,08	-0,91	-6,1	3	1	2	6	0	6
8	Mixt	-7,42		-4,85	-1,89	-6,9	7	5	2	6	12	6
9	Mixt	-7,42		-4,72	-1,77	-7	4	2	2	7	5	2
10	Mixt	-8,11		-5,1	-2,34	-7,8	2	4	2	5	12	7
11	Mixt	-7,13		-4,83	-2,38	-7,4	10	4	6	11	5	6
12	Mixt	-8,11		-5,17	-2,18	-8,1	0	7	7	7	7	0
								Average error:	4 ± 2		Average error:	5 ± 3

Table A3.3: Prediction of motor activity, ambulation and rearing counts of rats exposed to the mixture of Pb/As/Mn through its brain porphyrin levels, uro-, penta-, copro- and protoporphyrins. Data were analyzed by multiple linear regression. The values estimated by the model were compared with the number of movements observed in each rat.

Brain Porphyrin (P) Levels						Motor activity					
						Ambulation counts			Rearing counts		
Rat (code number)	Group	ln UroP	ln PentaP	ln CoproP	ln ProtoP	Predicted	Observed	Error	Predicted	Observed	Error
		(nmol/ g brain protein)									
7	Mixt	-4,68	-6,07	-5,65	-6,38	4	1	3	7	0	7
8	Mixt	-4,88	-6,21	-5,71	-6,38	6	5	1	9	12	3
9	Mixt	-5,71	-6,27	-6,21	-6,57	-2	2	4	4	5	1
10	Mixt	-5,17	-6,27	-6,17	-6,57	9	4	5	13	12	1
11	Mixt	-5,71	-6,21	-6,21	-7,01	4	4	0	3	5	2
12	Mixt	-4,68	-6,07	-5,65	-6,5	6	7	1	7	7	0
							Average error:	2		Average error:	2

Appendix 4

Table A4.1: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of Pb in blood, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

		Predicted type of exposure		
Group	N			
		CR	CU	M
CR	10	5	3	2
CU	12	0	12	0
M	13	2	0	11

80.0% of the cases correctly classified

Table A4.2: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of Mn in blood, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (group).

		Predicted type of exposure		
Group	N			
		CR	CU	M
CR	10	7	3	0
CU	12	4	8	0
M	13	2	3	10

71.4% of the cases correctly classified

Table A4.3: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of As in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

		Predicted type of exposure		
Group	N	CR	CU	M
CR	10	5	5	0
CU	12	7	5	0
M	13	0	4	9

54.3% of the cases correctly classified

Table A4.4: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of Pb in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

		Predicted type of exposure		
Group	N	CR	CU	M
CR	10	6	3	1
CU	12	4	8	0
M	13	7	3	3

48.6% of the cases correctly classified

Table A4.5: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of delta-ALA in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

Group	N	Predicted type of exposure		
		CR	CU	M
CR	10	6	1	3
CU	12	7	1	4
M	13	5	1	7

40.0% of the cases correctly classified

Table A4.6: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of uroporphyrins in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

Group	N	Predicted type of exposure		
		CR	CU	M
CR	20	4	7	9
CU	20	6	8	6
M	20	2	0	18

50.0% of the cases correctly classified

Table A4.7: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of heptaporphyrins in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

		Predicted type of exposure		
Group	N			
		CR	CU	M
CR	20	3	10	7
CU	20	2	14	4
M	20	1	18	1

30.0% of the cases correctly classified

Table A4.8: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through its individual levels of hexaporphyrins in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

		Predicted type of exposure		
Group	N			
		CR	CU	M
CR	20	16	0	4
CU	20	6	7	7
M	20	10	5	5

46.7% of the cases correctly classified

Table A4.9: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of pentaporphyrins in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

		Predicted type of exposure		
Group	N			
		CR	CU	M
CR	20	4	8	8
CU	20	1	13	6
M	20	3	2	15

53.3% of the cases correctly classified

Table A4.10: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of coproporphyrins in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

		Predicted type of exposure		
Group	N			
		CR	CU	M
CR	20	15	0	5
CU	20	7	5	8
M	20	4	0	16

60.0% of the cases correctly classified

Table A4.11: Identification of occupationally exposed subjects, miners (M), persons living in a urban environment (CU) and subjects from a rural area (CR) through their individual levels of protoporphyrins in urine, by discriminant analysis. The table represents the type of exposure predicted by the model versus its real exposure (Group).

		Predicted type of exposure		
Group	N	CR	CU	M
CR	20	19	1	0
CU	20	8	12	0
M	20	16	1	3

56.7% of the cases correctly classified

Appendix 5

Table A5.1: The procedure I' was tested subsequently in 7 subjects randomly selected to do not be included in the models. The table shows the real type of exposure, miners (M), urban (CU) and rural (CR) populations, the concentrations of urinary (U) As, Pb and ALA and the levels of Pb and Mn in blood (B) and the final result of the classification functions (CF) of each group [CF (CR)], [CF (CU)] and [CF (M)] (see Fig. x). The CF with the highest value corresponds to the type of exposure predicted by the model.

Subject (code)	Original group	As U (µg/g creat)	Pb U (µg/g creat)	ln ALA U (mg/g creat)	Pb B (µg/g blood)	Mn B (µg/g blood)	CF values	**Predicted type of exposure** *(highest CF value)*
I- H	CR	0.00	2.30	1.18	1.05	0.21	**CF (CR) = 18.2** CF (CU) = 17.6 CF (M) = -2.02	CR
II - H	CR	1.39	20.20	1.68	0.80	0.10	**CF (CR) = 16.20** CF (CU) = 14.40 CF (M) = -2.36	CR
III - H	CU	2.80	10.40	-0.80	1.19	0.15	CF (CR) = 20.6 **CF (CU) = 24.90** CF (M) = 8.05	CU
IV - H	M	9.31	19.88	-1.03	0.28	0.34	CF (CR) = -0.56 CF (CU) = -6.01 **CF (M) = 9.19**	M
V - H	M	0.96	23.58	0.05	0.67	0.23	**CF (CR) = 11.3** CF (CU) = 9.93 CF (M) = 2.25	CR
VI - H	M	17.99	18.60	-1,01	1.05	0.12	CF (CR) = 19.3 CF (CU) = 23.95 **CF (M) = 24.01**	M
VII - H	M	7.66	23.99	0,02	0.30	0.26	CF (CR) = 2.26 CF (CU) = -3.47 **CF (M) = 5.22**	M

One of seven subjects external to the model was wrongly identified as belonging to the rural population (CR), being its original classification occupationally exposed (M).