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BIOMONITORING OF ENVIRONMENTAL CONTAMINATION

RESULTING FROM MINING ACTIVITIES ON EXPOSED POPULATIONS

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CONTENTS

ORIGINAL PUBLICATIONS

The present work contains techniques and/or data also presented in the following scientific papers:

- **Coelho, P.**, García-Lestón, J., Costa, S., Costa, C., Silva, S., Fuchs, D., Geisler, S., Dall'Armi, V., Zoffoli, R., Bonassi, S., Pásaro, E., Laffon, B., and Teixeira, J.P. 2013. Immunological alterations in individuals exposed to metal(loid)s in the Panasqueira mining area, Central Portugal. *Arch. Toxicol. (submitted).*

- **Coelho, P.**, García-Lestón, J., Costa, S., Costa, C., Silva, S., Dall'Armi, V., Zoffoli, R., Bonassi, S., Lima, J., Gaspar, J., Pásaro, E., Laffon, B., and Teixeira, J.P. 2013. Genotoxic effects of environmental and occupational exposure to metal(loid)s. A molecular epidemiology survey of populations living and working in the Panasqueira mine area, Portugal. *Envir. Health Perspect. (submitted).*

- **Coelho, P.**, Costa, S., Costa, C., Silva, S., Walter, A., Ranville, J., Pastorinho, R., Harrington, C., Taylor, A., Dall'Armi, V., Candeias, C., Ferreira da Silva, E., Laffon, B., Bonassi, S., and Teixeira, J.P. 2013. Biomonitoring of several toxic metal(loid)s in different biological matrices from environmentally and occupationally exposed populations from Panasqueira mine area – Portugal. *Environ. Geochem. Health* (*accepted, in press*)

- **Coelho, P**., Costa, S., Costa, C., Silva, S., Walter, A., Ranville, J., Sousa, A., Coelho, M., Garcia-Lestón, J., Pastorinho, R., Laffon, B., Pásaro, E., Harrington, C., Taylor, A., and Teixeira, J. 2012. Metal(loid)s levels in biological matrices from human populations exposed to mining contamination – Panasqueira Mine (Portugal). *J. Toxicol. Environ. Health A* 75: 893–908.

- **Coelho, P.**, García-Lestón, J., Silva, S., Costa, C., Costa, S., Coelho, M., Laffon, B., Pasáro, E., and Teixeira, J.P. 2011. Geno- and Immunotoxic effects on populations living near a mine - Case study: Panasqueira mine. *J. Toxicol. Environ. Health A* 74: 1076-1086*.*

During these six years other scientific work was been published under to the same subject:

- **Coelho, P**., and Teixeira, J. P. 2012. Biomarkers, Human Health. In *Encyclopedia of Toxicology*, 3rd Edition, Elsevier (*in press*).

• **<u>Coelho, P.,</u>** Teixeira, J.P., and Gonçalves O. 2011. Mining activities: Health impacts. In *Encyclopedia of Environmental Health* edit by Nriagu J.O. Volume 3, pp. 788-802, Burlington: Elsevier.

• **<u>Coelho, P.</u>**, Silva, S., Roma-Torres, J., Costa, C., Henriques, A., Teixeira, J., Gomes, M., and Mayan O. 2007. Health impact of living near an abandon mine – Case study: Jales Mines. *Int. J. Hyg. Environ. Health* **210**: 399-402.

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LIST OF ABREVIATIONS

AMD Acid Mine Drainage

AP Apurinic/Apirimidinic

ARSC Administração Regional de Saúde do Centro

ATSDR Agency for Toxic Substances and Disease Registry

BER Base Excision Repair

BH4 Tetrahydrobiopterin

CA Chromosomal Aberrations

CBMN Cytokinesis Block Micronucleus test

CD Cluster of Differentiation

CI Confidence Interval

COFS Cerebro-Oculo-Facio-Skeletal

CRM Certified Reference Material

CS Cockayne's Syndrome

CYP Cytochrome P450

DDB1 DNA Damage-Binding Protein 1

DSB Double Strand Breaks

EDTA Ethylenediamine Tetraacetic Acid

ELISA Enzyme-Linked Immunosorbent Assay

Endo III Endonuclease III

EPA Environmental Protection Agency

ERCC Excision Repair Cross-Complementing

EU European Union

GPA Glycophorin A

GSH Glutathione

GST Glutathione S-Transferase

FISH Fluorescent *in situ* Hybridization

FITC Fluorescein-Isothiocyanate

FPG Formamidopyrimidine DNA Glycosylase

GTP-CH1 Guanosine-Triphosphatecyclohydrolase-1

HLA Human Leukocyte Antigen

HMS Heavy Metal Separation

HPLC High Performance Liquid Chromatography

HPRT Hypoxanthine-guanine Phosphoribosyltransferase

HR Homologous Recombination

IAEA International Atomic Energy Agency

IARC International Agency for Research on Cancer

ICP-MS Inductively Coupled Plasma-Mass Spectrometry

ICP-OES Inductively Coupled Plasma-Optical Emission Spectrometry

IDO Indoleanine-2,3-dioxygenase

IFN Interferon

Ig Immunoglobulins

IL Interleukin

INETI Instituto Nacional de Engenharia, Tecnologia e Inovação

LOD Limit of Detection

MHC Major Histocompatibility Complex

MMR Mismatch Repair

MR Mean Ratio

NER Nucleotide Excision Repair

NHEJ Non-Homologous End Joining

NIES National Institute of Environmental Sciences

NK Natural Killer

MN Micronuclei

NO Nitric Oxide

NOS Nitric Oxide Synthase

NRC National Research Council

PBS Phosphate Buffer Solution

PCNA Proliferating Cell Nuclear Antigen

PCR Polymerase Chain Reaction

PE Phycoerytrin

PE-Cys Phycoerytrin-Cyanin5

PEEK Polyether Ether Ketone

PNAAS Plano Nacional de Acção Ambiental em Saúde

QC Quality Control

ROS Reactive Oxygen Species

SCE Sister Chromatid Exchange

SCGE Single-Cell Gel Electrophoresis

SD Standard Deviation

SDSA Synthesis-Dependent Strand Anneling

SNPs Single Nucleotide Polymorphisms

SSB Single Strand Breaks

Tc T-cytotoxic lymphocytes

TCR T-Cell Receptor

TCR-Mf TCR Mutation frequency

Th T-helper lymphocytes

TL Tail Length

TM Tail Moment

TTD Trichothiodystrophy

WHO World Health Organisation

XP Xeroderma Pigmentosum

%DNAT Percentage of DNA in the tail (tail Intensity)

ABSTRACT

Mining industry is a vital economic sector for many countries but it is also one of the most hazardous activities, both occupationally and environmentally. During these processes several toxic wastes are produced and released into the surrounding environment causing pollution of air, drinking water, rivers and soils. Mining activities cause several health impacts in miners and communities living near the mine site that may persist, even when the mine is abandoned. Major impacts on workers' health are cancer and respiratory diseases such as asbestosis, silicosis, and pneumoconiosis.

The Panasqueira mine (Sn-W) in Central Portugal was selected as the study area due to its past and current activity, and its potential impact on the local ecosystems. There are small villages around the mine site, namely S. Francisco de Assis and Barroca do Zêzere, with local populations strongly dependent on agriculture and farming. Another important factor was Zêzere river, which feeds the Castelo do Bode dam (located 90 Km downstream from the mine), the principal water supply of Lisbon metropolitan area. Any significant spillage into this river can bring serious environmental consequences to regional economy importance. From the results obtained in geochemical sampling campaigns, it was concluded that a significant geochemical dispersion, with anomalous distribution of some metal(loid)s, occurs in the study area.

The aim of this project was to evaluate the impact of environmental metal(loid)s contamination in populations living nearby and working in Panasqueira mine, through a multistage approach that integrates information obtained with biomarkers of exposure, effect and susceptibility. The possible health effects caused by this environmental contamination were also discussed.

Study group consisted of 41 individuals environmentally exposed, 41 individuals occupationally exposed and 40 individuals without known exposure to metal(loid)s (controls). Biomarkers of exposure included quantification of several metal(loid)s - As, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Se, S, Si e Zn - in blood, urine, nail (finger and toe), and hair samples by inductively coupled plasma-mass spectrometry (ICP-MS) and inductively coupled plasma-optical emission spectrometry (ICP-OES). Several biomarkers of effect were analyzed, such as, cytogenetic techniques – micronucleus (MN) test and chromosomal aberrations (CA); comet assay, T-cell receptor (TCR) mutation assay, alterations in lymphocyte subsets percentages (CD3$^+$, CD4$^+$, CD8$^+$, CD15$^+$56$^+$) and quantification of neopterin, tryptophan, kynurenine and nitrite. Finally several genetic polymorphisms in genes involved in the metabolic pathway of metal(loid)s (*GSTA2*, *GSTM1*, *GSTP1*, and *GSTT1*) and in genes involved in DNA damage repair (*XRCC1*,

APEX1, *MPG*, *MUTYH*, *OGG1*, *PARP1*, *PARP4*, *ERCC1*, *ERCC4*, and *ERCC5*) were evaluated as biomarkers of susceptibility.

Results obtained in biomarkers of exposure agree with the reported by the environmental studies performed in this area pointing to populations living nearby and working in the mine being exposed to metal(loid)s origination from mining activities. Arsenic was the element with the highest increase in exposed populations. The concentration of other elements such as Cr, Mg, Mn, Ni, Pb, S, Se, and Zn also increased, although at a lesser extent, especially in individuals with environmental exposure and in females. Significant increases in the frequency of all the biomarkers of effect investigated (TCR mutation, CA, MN, and DNA damage measured by the comet assay) were found in both exposed groups, generally higher in those environmentally exposed. The environmentally exposed group also showed significantly lower levels of %CD8$^+$ and higher CD4$^+$/CD8$^+$ ratios, whereas the occupationally exposed individuals showed significant decreases in %CD3$^+$ and %CD4$^+$, and significant increases in %CD16$^+$56$^+$, when compared to controls. Allele frequencies of studied polymorphic genes obtained in this study were similar to the ones described by other authors for Caucasian populations. Significant influences of polymorphisms were observed for *GSTM1* deletion and *OGG1* rs1052133 on CA frequencies, *APEX1* rs1130409 on DNA damage, *ERCC1* rs3212986 on DNA damage and CA frequency, and *ERCC4* rs1800067 on MN and CA frequencies.

Our results show that the metal(loid) contamination in the Panasqueira mine area induced genotoxic damage both in individuals working in the mine or living in the area. The observed effects are closely associated to the internal exposure dose, and are more evident in susceptible genotypes. The contamination is also inducing immunotoxic effects in exposed populations which can lead to a complete deregulation of the immune system increasing populations' susceptibility to many pathologies.

All these findings confirm the need for competent authorities to act as soon as possible in this area and implement strategies aimed to protect exposed populations and the entire ecosystem.

RESUMO

A exploração mineira produz ao longo dos anos um conjunto assinalável de impactes negativos no ambiente, com consequentes efeitos na saúde das populações residentes na envolvência. Adicionalmente a atividade mineira continua a ser uma das profissões mais perigosas do mundo, sendo os principais efeitos na saúde dos trabalhadores as doenças oncológicas e respiratórias como a silicose e pneumoconiose.

A escolha da envolvente da mina da Panasqueira (Sn-W) como objeto de estudo deste projeto reside nos seguintes fatores: (a) trata-se de uma exploração mineira em plena actividade; (b) apresenta escombreiras de volume avultado, bem como barragens de estéreis; (c) a mina coexiste com a presença de pequenas povoações na sua vizinhança; (d) os rejeitados encontram-se nas proximidades do rio Zêzere que alimenta a barragem do Castelo de Bode, a principal fonte abastecedora de água da cidade de Lisboa. Estudos anteriores feitos nesta região, no âmbito de um projeto comunitário, revelaram existir uma significante dispersão de metais/metaloides, com assinaturas geoquímicas anómalas a distâncias consideráveis nos sedimentos ao longo do Rio Zêzere.

O objetivo do presente projeto foi avaliar o impacto da contaminação ambiental por metais/metaloides em populações residentes na vizinhança e trabalhadores da mina da Panasqueira, uma abordagem múltipla de forma a integrar a informação obtida na análise de biomarcadores de exposição, efeito e susceptibilidade. Os possíveis efeitos na saúde causados por esta contaminação foram também discutidos.

A população em estudo consistiu em 41 indivíduos ambientalmente expostos, 41 indivíduos ocupacionalmente expostos e 40 indivíduos controlo sem histórico de exposição a metais/metaloides. Os biomarcadores de exposição estudados incluíram a quantificação de diversos metais/metaloides – As, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Se, S, Si e Zn - em amostras de sangue, urina, unhas (pés e mãos) e cabelos por espectrometria de massa com plasma indutivamente acoplado (inductively coupled plasma-mass spectrometry - ICP-MS) e espectrometria de emissão óptica com plasma indutivamente acoplado (inductively coupled plasma-mass spectrometry - ICP-OES). Diversos biomarcadores de efeito foram analisados, nomeadamente: técnicas citogenéticas - teste do micronúcleo (MN) e aberrações cromossómicas (AC) -, teste do cometa, teste de mutação do receptor das céluals T (TCR), alterações nas percentagens de subpopulações linfocitárias (CD3$^+$, CD4$^+$, CD8$^+$, CD15$^+$56$^+$) e quantificação de neopterina, triptofano, quinurenina e nitrito. Por último como biomarcadores de susceptibilidade foram estudados polimorfismos de genes relacionados com o metabolismo de metais/metaloides (*GSTA2*, *GSTM1*, *GSTP1* e *GSTT1*) e polimorfismos

de genes envolvidos no mecanismo de reparação de lesões do DNA (*XRCC1, APEX1, MPG*, *MUTYH*, *OGG1*, *PARP1*, *PARP4*, *ERCC1*, *ERCC4* e *ERCC5*).

Os resultados obtidos para os biomarcadores de exposição são concordantes com os relatados nos estudos ambientais efetuados nesta área e que indicam que as populações residentes na vizinhança e os indivíduos que trabalham na mina estão expostos a metais/metaloides com origem nas atividades mineiras. O Arsénio foi o elemento que apresentou maior aumento nas populações expostas quando comparadas com a populações controlo. Também se verificou o aumento de outros elementos tais como Cr, Mg, Mn, Ni, Pb, S, Se, e Zn, apesar de ser a um nível mais baixo, nomeadamente em indivíduos ambientalmente expostos e mais especificamente em indivíduos do sexo feminino. Foram também observados aumentos nas frequências de todos os biomarcadores de efeito estudados (mutações no TCR, AC, MN e dano no DNA detectado através do teste do cometa) em ambos os grupos expostos quando comparados com o grupo controlo, sendo no geral mais elevados nos indivíduos expostos ambientalmente. Relativamente aos biomarcadores de imunotoxicidade foi observada uma diminuição significativa nas percentagens de CD8$^+$ e um aumento significativo na razão CD4$^+$/CD8$^+$ no grupo ambientalmente exposto e uma diminuição significativa nas percentagens de CD3$^+$ e CD4$^+$ e um aumento significativo nas percentagens de CD16$^+$56$^+$ no grupo ocupacionalmente exposto. As frequências alélicas dos genes polimórficos estudados são similares às descritas noutros estudos para as populações caucasianas. Foram detetadas influncias significativas de diversos polimorfismos nos biomarcadores estudados, nomeadamente dos polimorfismos do *GSTM1* e do *OGG1* rs1052133 nas frequências de AC, do *APEX1* rs1130409 no nível de dano no DNA, do *ERCC1* rs3212986 no nível de dano no DNA e nas frequências de AC, e finalmente do *ERCC4* rs1800067 nas frequências de MN e AC.

Os resultados obtidos mostram que a contaminação por metais/metaloides na área da mina da Panasqueira induziu dano genotóxico tanto em indivíduos que residem na vizinhança da mina como nos que nela trabalham. Os efeitos observados estão directamente associados com a dose interna, e são mais evidentes em genótipos mais susceptíveis. A contaminação induziu também efeitos imunotoóxicos nas populações expostas podendo levar a uma desregulação do sistema imunitário aumentando assim a suscetibilidade das populações a diversas patologias.

Globalmente estes resultados confirmam a necessidade de atuação imediata das autoridade competentes nesta área e a implementação de estratégias que visem a proteção das populações expostas e de todo o ecossistema.

I. REVIEW OF THE LITERATURE

1. INTRODUCTION

After centuries of economic and social development without concern for contamination of the environment, in recent decades several measures for the adoption of sustainable development models that safeguard the environment have been developed. Studies on the relationship environment/health estimate that every year, the premature death of thousands of citizens can be attributed to environmental factors (OECD, 2008). Human health depends in an essential way on the environment as both a source of resources and a deposit for wastes. Environmental impact assessment is a crucial tool for maintaining and improving environmental quality while carrying out economic development (Health Canada, 2004).

Considering that health has a crucial role in the context of sustainable development, the European Union (EU) has prepared some contingency plans. The European Environment and Health action plan 2004-2010, approved on 9th of June 2004, formed the basis of the specific programs that each country should establish. A central objective of this plan was to obtain the information needed to reduce the adverse health effects of environmental pollution. Among the highest priorities was the issue of heavy metals and cancer disease particularly in children. It was also highlighted the role of biological monitoring as a fundamental tool to assess exposure. Portugal only in May 2007 presented a proposal from the National Action Plan for Environment and Health (PNAAS – *Plano Nacional de Acção Ambiente em Saúde*) which was approved on the 4th of June 2008, in the Resolution of the Council of Ministers 91/2008, and will take place until 2013. This project was intended to be included within the scope of this program.

2. MINING ACTIVITIES: HEALTH IMPACTS

Mining is one of the oldest activities in human civilization. Mining industry is a vital economic sector for many countries, but is also one of the most hazardous activities in occupational and environmental context. Nowadays, ecosystems as well as populations in the surroundings of mining areas remain exposed to toxic levels of pollution due to an ineffective requalification of these areas, not only after the ceasing of exploitation, but also during the exploitation process (Coelho et al., 2007).

Investment in improving the health of communities and workers affected by mining activities is commendable not only to decrease the current exposure and risk, but also to reveal the need for changes in mining laws and regulations.

Environmental impact assessment involves the determination of changes or impacts that a project or an action will have on the surroundings (positive or negative effects) before it is carried out to prevent irreversible damages (Coelho et al., 2011a). Most studies report several adverse effects caused by mining wastes at all levels of organization, from cellular to ecosystem level. These studies allow establishing a cause-effect relationship between mining activities and the accumulation of trace elements at concentrations that are potentially harmful to all organisms' health (Peplow and Edmonds, 2005).

The impact of mining activities on the community health occurs at various levels, namely adverse health effects that result from environmental exposure to contaminated air, water, soil, and noise pollution. Existing studies point to several adverse effects on communities' health, such as mesothelioma, and respiratory illness, but most of the results reported are conflicting (Stephens and Ahern, 2001). It is worth noting that many adverse effects are only noticed in groups of risk, such as children and elder people, since their health is more likely to be affected. Moreover, long-term effects are a very important issue, which must be taken into account.

In what concerns occupational impacts, mining continues to be one of the most hazardous professions in the world. Short-term injuries and fatalities occur at high rates, but also long-term impacts. Long-term effects are particularly relevant in miners who have worked in asbestos, coal and uranium mines or in miners who have been exposed to a mixture of different silica and other dusts (Stephens and Ahern, 2001). Workers of deep mines are exposed to more hazardous situations than those working in open cast mines, due to the risk of collapse, poor air quality and underground explosions (Coelho et al., 2011a). Noise, vibration, dust, heat and humidity are some of the factors affecting them. Major impacts on workers' health are cancer and respiratory diseases such as asbestosis, silicosis, and pneumoconiosis. Cancer is a long-term impact and it is one of the most important occupational health effects as miners can develop health problems many years after they finish working (Stephens and Ahern, 2001).

3. PANASQUEIRA MINE: STUDY AREA

3.1. Site description

The Panasqueira mine is located in the municipalities of Covilhã and Fundão (Castelo Branco district, in central Portugal - 40º9'57" N, 7º45'37" W) – **Figure 1a)**. The mining district is situated between the Gardunha and S. Pedro de Açor mountains, to the west of Central Portugal depression Cova da Beira (Beira Baixa province). The topography

ranges in altitude from 350 to 1080 m (Reis, 1971), with deep valleys. The average annual rain precipitation in the region is 1200-1400 mm; snow fall is frequent, particularly above 700 m. The average annual temperature is ≈ 12° C, ranging values from ≈ 0 °C during the winter to about≈ 30ºC in summer. The evapotranspiration in this region is around 1080 mm (source www.apambiente.pt/). The streams are generally dry in the summer and flooded in the winter.

Panasqueira mine was selected as a target area of our project due to its past and current activity, giving rise to large visual impact and also in the local ecosystems. There are small villages around the mine site, namely S. Francisco de Assis (40º8'34" N, 7º44'9" W) and Barroca do Zêzere (40º6'40'' N, 7º43'1'' W), with local populations strongly dependent of land use and water for agriculture, drinking and cattle breeding (**Figure 1b**). Another important factor driving such choice is that the main river flowing in the area (Zêzere river) feeds the Castelo do Bode dam (located 90 Km downstream the mine - **Figure 1a**), which is the principal water supply of Lisbon metropolitan area (where about 1/3 of Portuguese population lives). Any significant spill occurring in Rio tailing (old tailing located near Zêzere river – **Fig. 1b**) can bring serious environmental consequences to extensive areas with significant regional economic importance.

a)

b)
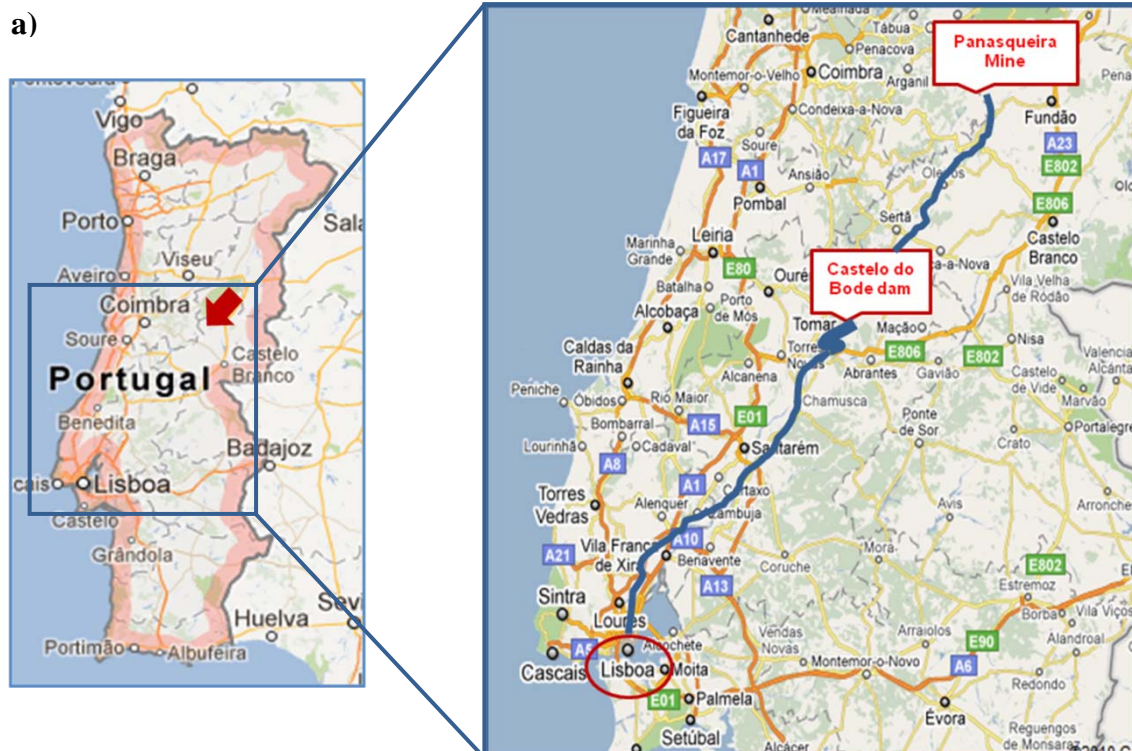
FIGURE 1 – **(a)** Location of Panasqueira mine (red arrow on the left map), the Castelo do Bode dam, and the Zêzere river flow (blue line across the map on the right); **(b)** Map showing the location of Rio tailing (~1,200,000m^3) and mud impoundment (~731,034m^3) (orange circle), Barroca Grande tailing (~7,000,000m^3) and two mud impoundments (~1,193,885m^3) (yellow circle) and several small villages nearby these structures (red squares: the one in the upper left corner is S. Francisco de Assis; the other three on the bottom belong to Barroca do Zêzere and Pampilhosa da Serra parishes). One can also see Zêzere river flowing from the middle right side, close to Rio tailings where it merges with a small river (Casinhas creek) coming from upstream Barroca Grande and that passes through S. Francisco de Assis, and then flows down nearby the villages from Barroca do Zêzere and Pampilhosa da Serra parish. Image adapted from Candeias (2010).

3.2 Geology and mineralization

According to the paleogeographic and tectonic zoning established for the Hercynian Chain of the Hesperic massif (Julivert et al., 1974; Lotze, 1945), this deposit is included in the Central Iberian Zone. As a result of the regional metamorphism, the sediments changed to biotite-chlorite schists and phyllites, and the more arenaceous units converted to dark,

fine-grained quartzites. The Panasqueira deposit lies in a folded metasedimentary sequence, the Beira-Schist Formation of upper Precambrian-Cambrian age. The Beira Schist Formation was subjected to lower greenschist grade regional metamorphism during the early compressive stages of the Hercynian Orogeny. The Beira-Schist Formation is composed of a several thousand metre thick sequence of lower marine flyschoid schists, greywackes, lenticular, thinly bedded mudstones, shales, and arenites (Bloot and de Wolf, 1953; Cavey and Gunning, 2006; Conde et al., 1971; Kelly and Rye, 1979).

The dominant lithological unit hosting the tin-tungsten concessions of the Beralt tin & wolfram Company was of Cambrian to Upper Cambrian age, and forms an outcrop of about 35 km^2; however, brown argillaceous schists and dark grey siliceous schists interbedded with rare greywackes were identified in the area. Few mafic rocks (dolerites) were observed, particularly near S. Jorge da Beira (NW-SE subvertical). Some quartz veins disposed parallel to the principal schistosity were also mapped. An important 4 m thick, N65ºE vertical fault, the Ribeira da Cebola fault, was observed between the villages of S. Jorge da Beira and Panasqueira. It is characterised as a sinistral fault that caused a horizontal displacement of about 95 m on the schist formation.

The Panasqueira ore deposit is a typical example of a Sn-W hydrothermal mineralization associated with the Hercynian plutonism. The mineralized zone has an average length of 2500 m, ranges in width from 400 to 2200 m and extends at least 500 m in depth (Cavey and Gunning, 2006). The Panasqueira mine has been in production for over 100 years and is one of the largest economic vein deposits in the world. During the period 1947-2001, over 27 million tonnes of rock has been mined, which has produced approximately 92.8 thousand tonnes of tungsten concentrate, 4.8 thousand tonnes of tin concentrate and 28.6 thousand tonnes of copper concentrate (Smith, 2006).

The granitic intrusion is thought to be the principal source of the mineralizing fluids responsible for the economic wolframite vein system. The most dominant and important structural feature at Panasqueira mine is a flat open joint system that is prevalent throughout the mine workings. The remobilized ore bearing fluids migrated from the intrusion into these flat joints to form the stacked quartz vein system being mined today. A second set of non-wolframite bearing quartz veins (veins contain minor chalcopyrite, galena, and pyrite) also exists at the Panasqueira deposit, and are aligned with the vertical foliation and cut by the later tungsten-bearing hydrothermal vein system (Cavey and Gunning, 2006).

The Panasqueira deposit consists of a series of stacked, sub-horizontal, hydrothermal quartz veins intruding into the Beira schists and shales. The paragenesis is complex,

nevertheless four stages of mineral formation are generally accepted by most of the authors who have studied this deposit: 1) oxide-silicate phase [quartz, wolframite; cassiterite]; 2) main sulphide phase [pyrite, arsenopyrite, pyrrothite, sphalerite, chalcopyrite]; 3) pyrrothite alteration phase [marcasite, siderite, galena, Pb-Bi-Ag sulphosalts]; 4) late carbonate phase [dolomite, calcite] (Breiter, 2001; Correa and Naique, 1998; Corrêa de Sá et al., 1999; Noronha et al., 1992). At Panasqueira, more than 65 minerals, including sulphides, sulphosalts, oxides, carbonates, silicates, phosphates, and tungstates minerals, have been identified (Kelly and Rye, 1979).

In general, the most common minerals in addition to quartz are: wolframite, pyrite, pyrrhotite, arsenopyrite, chalcopyrite, cassiterite, beryl, mica, and fluorite. The Panasqueira mine has many rare minerals that can only be observed microscopically, making it a site of specific scientific importance.

3.3 Mining activity and potential environmental considerations

Historical data show that Romans worked the area for tin. The first prospecting licence was granted in 1886 and the first reference to wolframite mineralization in Panasqueira mine reportedly dated from 1888 (Cavey and Gunning, 2006). The Panasqueira mining concession covers an area of more than 2000 ha and developed through the amalgamation of a Portuguese company. In 1928, the concession was taken over by Beralt Tin and Wolfram Lda. (Corrêa de Sá et al., 1999; D'Orey, 1967). The first underground drifts were opened at Cabeço do Pião (Rio), but mining activity decreased there as richer veins were discovered at the Panasqueira site.

The exploitation was extended to other areas, the most important being Barroca Grande, Corga Seca, Panasqueira, Rebordões, and Vale da Ermida. The economic exploitation has mainly focused on wolframite, cassiterite, and chalcopyrite, the last two as by-products. On average the "tout-venant" contains 0.3% WO_3 and the three final concentrates are recovered with 75% WO_3, 72% SnO_2, and 22% Cu together with significant amounts of Ag. At the present time, exploitation is focused at Barroca Grande. Panasqueira is an underground mine using the room and pillar mining methods.

The long history of exploitation and ore treatment operations are testified by the presence of tailings and other debris disposed of in the Vale de Ermida. The site is characterised by the presence of old infrastructures as well as large tailings piles from the earliest years of production (late 1890s to 1927) that were abandoned when the new plant was constructed at Rio (Cavey and Gunning, 2006). At that time, the ore was transported from Barroca Grande along a 4 km cableway further down the river Zêzere to the concentration plant

located in Rio. The Rio plant and tailings disposal area were deactivated in 1996; nowadays, the waste rock is still placed in the Barroca Grande tailings area (D'Orey, 1967).

During the mining process, two types of mine waste are generated: coarse aggregates derived from rock blasting, which is in fact a by-product used in minor quantities in hot bituminous mixtures since 1970s or disposed in tailings; and waste mud coming from the plant and conveyed by pipelines into lagoons built specifically for that purpose (several million tonnes have been deposited and almost 100 tonnes are being added each day).

The ore treatment process begins with heavy media separation (HMS) for the coarse fractions of material. In a second phase, cyclones are used to produce ore concentrates with high metal content, and tables are used to treat the sands. These pre-concentrates contain all the existent heavy minerals, such as wolframite, cassiterite, sulphides, and siderite. Until 1996, these pre-concentrates were transported to the Rio plant, but today, the final separation procedures are carried out exclusively in Barroca Grande. James flotation tables are used to float the pre-concentrates; the floated sulphides constitute the steriles, which feed the copper circuit. Copper concentrates are recovered after differential flotation in Denver cells (Corrêa de Sá et al., 1999; Reis, 1971; Rodrigues Abrantes and Gonzalez, 1973). It must be emphasised that arsenopyrite (the main sulphide present) is rejected with the tailings, which contain about 30% As.

The Rio tailing site, located near and draining directly to the Zêzere River, is a large site where tailings have been disposed of for approximately 90 years. This deposit has an average height of 90 m and has slopes of about 35º. The grain size of the materials is variable, due to successively finer grinding procedures, and are classified as: coarse sterile material from the mine, coarse tailings (sterile material) from the heavy media separation, sand, mud, and slush. The three first types of materials are disposed of in the tailings, while the mud and slush were discharged into a pond that was intentionally created for that purpose (SE section of the tailings site). The existing pond at the Rio tailings dam is an open-air impoundment and contains 731034 m^3 of rejected ore concentrates with high metal levels. At the far other end of the tailings, an arsenopyrite stockpile, with 9400 m^3, had been deposited in the past, and remained exposed until June 2006, when the pile was capped with geotextile and layers of clay (e-EcoRisk, 2007). There are several slippage zones evident in the tailings, indicating a risk of collapse. In this scenario, the arsenopyrite stockpile will enter directly into the Zêzere River. When this pile was exposed (prior to June 2006) to the atmosphere and rain water, the oxidised materials generated acid mine drainage (AMD).

The Barroca Grande site includes underground mine and portals, a processing plant, mine offices, and employee housing, in addition to the active tailings disposal areas, and the Salgueira water treatment plant (Cavey and Gunning, 2006). A huge tailings pile and two mud dams exist at this site. One of the dams is old and deactivated, although stabilized in geotechnical terms, whilst the other (smaller and disposed over the tailings) is still being fed with steriles (some rich in sulphides) obtained from the ore dressing operations. These tailings and impoundments are exposed to the atmospheric conditions. Surface runoff and water percolation leach the tailings and form AMD. The tailings piles at Barroca Grande are adjacent to the small, but perennially flowing, Casinhas stream, which drains to the Zêzere River. The Salgueira water treatment plant receives surface water from the old tailing pond area, water from the new tailings pond, mine drainage water, and seepage from the base of the tailings. These waters are mainly treated with lime. The precipitated sludge is pumped to the tailings pond while the treated water is pumped into holding tanks for later use in the mill or discharged to the creek channel adjacent to the plant and discharged into the Zêzere River.

3.4 Environmental studies: main results

Panasqueira Mine was one of the two Portuguese test sites studied in the scope of the e-EcoRisk project (between 2002 and 2007). This is the biggest study preformed in this area. From the results achieved in the geochemical sampling campaigns, it was concluded that a significant geochemical dispersion, with anomalous patterns, occurs downstream Barroca Grande tailings. These results also identified the anomalous distribution of several metals and metalloids in stream sediments and surface waters collected in local streams, and also in soil samples from nearby villages (Ávila et al., 2008; Grangeia et al., 2011; Salgueiro et al., 2008). Furthermore, when comparing the mean values of Casinhas stream (the small river flowing through S. Francisco de Assis) and Zêzere rivers with the mean values of the background stream sediments, it was apparent there was a moderate to strong enrichment of As (157✗; 26✗) and Cd (34✗; 59✗) (Ávila et al. 2008) enrichment when compared with the geochemical background (ratios values: Casinhas∕Backgroud; Zêzere River∕Background).

Recently (since 2010) a group from the Department of Geosciences - University of Aveiro (GeoBioTec) and the National Laboratory of Energy and Geology (LNEG) is collecting and analyzing different types of samples, such as road dusts, soils, vegetables for human consumption, superficial and groundwaters and stream sediments. Their preliminary

results agree with the ones from the previous study reporting extremely high concentrations of metals and metalloids in all these matrices (personal communication).

4. METAL(LOID)S

In 1981 Mertz defined trace elements as chemical elements found in our body at very low concentrations. Some of them are necessary for growth, development and proper biological function; therefore are essential. Essential elements include Cr, Cu, I, Mo, Se, and Zn. Some of them act as cofactors for various enzymes involved in essential cellular functions.

Trace metals/metalloids [=metal(loid)s] are a subclass of trace elements. They are a necessary part of nutrition and physiology; however exposure to high quantities is often toxic (Murray et al., 2009). Some of the elements included in this subclass are As, Cd, Cr, Cu, Fe, Hg, Mn, Pb, Se, and Zn.

4.1 Human exposure

Environmental and occupational exposure to metal(loid)s is a reality worldwide, though with different contours, affecting a significant number of individuals. One of the situations where conditions are gathered at highest risk of exposure is the mining context.

Water pollution problems by mining activities include AMD which is one of the most important environmental impacts. Highly acidic water solubilises metal(loid)s - Al, As, Cd, Cu, Pb, Ni, and Zn - carrying them into local ground and surface waters. This causes a major problem either through the consumption of fish and other biota that bioaccumulate metal(loid)s, through drinking water that wasn't treated in such a way to eliminate these elements, and also through dermal contact (Coelho et al., 2011a).

Mining processes can also result in the contamination of sediments in local streams. Sediments, coming from increased soil erosion, cause siltation or the smothering of streams beds. This siltation affects fisheries, swimming, domestic water, irrigation, and other uses of streams. Some toxic constituents associated with discharges from mining operations (i.e., Hg and P) may be found at elevated levels in sediments. Sediment contamination provides a long-term source of pollutants through potential redissolution in the water column (Coelho et al., 2011a). This may lead to chronic contamination of water and aquatic organisms

Particulate matter is one of the main problems, both occupationally and environmentally. Workers are highly exposed to them due to poor air quality inside the mines. Environmental exposure is also a key issue as it is released when overburden is stripped from the site and stored or returned to the pit. When the soil is removed, vegetation is also removed, exposing the soil to the weather, causing particulates to become airborne through wind erosion and road traffic. Particulate matter can be composed of toxic materials such as metal(loid)s like As, Cd, and Pb. In general, particulates affect human health adversely by contributing to illness related to the respiratory tract, such as emphysema, but they can also be ingested or absorbed through the skin (dermal contact) (Coelho et al., 2011a). These are the main routes of exposure in occupational settings.

Gaseous emissions are also important since some of them (e.g. sulfur oxide) affect the downwind environments through acid precipitation or dry deposition. Some metal(loid)s like As, Cd, Hg, and Zn vaporize when heated in pyrometallurgical processes, and if they are not captured and condensed, they affect firstly miners (when not properly equipped) and secondly the surrounding environment.

Table 1 presents a summary of the major environmental and human sources of exposure, and consequent effects of some of the most toxic metal(loid)s according to the International Agency for Research on Cancer (IARC) and the Agency for Toxic Substances and Disease Registry (ATSDR) classification.

Table 1. Main toxic metal(loid)s found in mining environments, indicating their main sources and effects. Adapted from Coelho et al. (2011a).

METAL (IARC / ASTDR CLASSIFICATION)*	ENVIRONMENTAL SOURCES	ENVIRONMENTAL EFFECTS	HUMAN EXPOSURE	HEALTH EFFECTS
Arsenic (Group 1A / 1st)	Arsenic can be found naturally on earth in small concentrations. May enter air, water and land through wind-blown dust and water run-off. Large amounts of arsenic end up in the environment and in living organisms due to volcanoes, microorganisms and human activities such as mining and agriculture.	It cannot be destroyed once it enters the environment. Large amounts added can spread and cause adverse health effects to humans and animals. High concentrations of arsenic may be found in plants as they absorb arsenic easily. Plant-eating freshwater organisms accumulate arsenic in their bodies and may affect the animals higher up the food chain.	Humans can be exposed through food (fish and seafood), water and air. It may also occur through dermal contact with soil or water. Exposure may be higher for people who work with arsenic compounds, drink significant amounts of wine, smoke, live near a mining site, and for those living on farmlands where arsenic-containing pesticides have been applied in the past.	Arsenic is one of the most toxic elements. Exposure to it can cause several health effects, namely irritation of the stomach, lungs and intestines, decreased production of red and white cells, skin changes. Significant uptakes of inorganic arsenic can lead to cancer development, especially skin, lung, liver and lymphatic cancers. Higher exposure can cause infertility and miscarriages, skin disturbances, declined resistance to infections, heart disruptions and DNA damage. High exposure to organic arsenic can cause nerve injury and stomachaches.
Cadmium (Group 1 / 7th)	Cadmium can mainly be found in the earth's crust. Large amounts of cadmium are naturally released in the environment, namely in rivers through weathering of rocks, into air through forest fires and volcanoes. Human activities such as mining also release significant amounts of cadmium in the environment. Soils are the main final destination of the industrial cadmium wastes. Other important source of cadmium in soils	Acidified soils enhance cadmium uptake by plants and this causes a potential danger to the animals which feed on them, and to the rest of the food chain. Earthworms and other essential soil organisms are extremely susceptible to cadmium poisoning. High concentrations of cadmium in soils can threaten the whole soil ecosystem.	Human uptake of cadmium occurs through food ingestion (liver, mushrooms, shellfish, mussels, cocoa powder and dried seaweed). Exposure to high concentrations of cadmium takes place through tobacco smoke. Cadmium is transported into lungs and then distributed through the whole body.	Breathing in cadmium can severely damage the lungs and, in last instance, can cause death. Cadmium is transported to the liver where it is bound to proteins to form complexes that are transported to the kidneys. There, it accumulates and damages filtering operations causing the excretion of essential proteins and sugars from the body. Excretion of bioaccumulated cadmium from the kidney takes a long time. It may cause damages in liver,

	is the appliance of artificial phosphate fertilizers in farmlands. Waste combustion and burning of fossil fuels are a main source of cadmium in the air. Only small amounts of cadmium are released in water through disposal of wastewater from households and industries.	Cadmium may bioaccumulate in several aquatic organisms (mussels, oysters, shrimps, lobsters, fish, etc.). Salt-water organisms are known to be the most resistant. Animals exposed to cadmium commonly present high blood-pressures, liver disease and nerve or brain damage.	Other high exposures occur with people who live near hazard waste sites or factories that release cadmium into the air and people who work in refinery industry.	diarrhea, stomach pains and severe vomiting; bone fracture, reproductive failure and possibly even infertility, damage to the central nervous and immune system, psychological disorders and it may also cause DNA damage leading to cancer development.
Chromium (Group 3 / 78th) Chromium (IV) (Group 1 / 17th)	Chromium enters the air, water and soil in the chromium (III) and chromium (VI) forms through natural processes and human activities. The main human activities that increase the concentrations of chromium (III) are steal, leather and textile manufacturing. As for chromium (VI) main activities are chemical, leather and textile manufacturing, and electro painting. These applications mainly increase concentrations of chromium in water. Through coal combustion chromium ends up in air, and through waste disposal chromium ends up in soils. Most of the chromium in air eventually settles in waters or soils. Chromium in soils strongly attaches to soil particles and as a result it moves towards groundwater. In water chromium absorbs on sediment and become immobile.	Most plants have adapted their systems to control chromium uptake (to be low enough not to cause any harm). But when the amount of chromium in the soil is extremely high, and the soils are rather acidic, uptake of high concentrations occurs. Plants usually absorb only chromium (III). Chromium is not known to accumulate in fish, but high concentrations of chromium in surface waters can damage their gills. In animals chromium can cause respiratory problems, a lower ability to fight disease, birth defects, infertility and tumor formation.	Humans can be exposed to through breathing, eating/drinking, and through dermal contact with chromium or chromium compounds. Exposure to chromium (VI) can occur in workers of steel and textile industries. Other sources of exposure are contaminated well water and tobacco smoke. For most people eating food that contains chromium (III) is the main route of chromium uptake, as it occurs naturally in many vegetables, fruits, meats, yeasts and grains. Various ways of food preparation and storage may alter the chromium contents of food. When food in stores in steel tanks or cans chromium concentrations may rise.	The health hazards associated with exposure to chromium are dependent on its oxidation state. Chromium (III) is an essential nutrient for humans but the uptake of too much chromium (III) can cause health effects as well, for instance skin rashes. Chromium (VI) is known to cause various health effects, such as skin rashes, upset stomachs and ulcers, respiratory problems, weakened immune system, kidney and liver damage, alteration of genetic material, and lung cancer.

Lead (Group 2A / 2nd)	Lead occurs naturally in the environment but most of what is found results from human activities such as mining and agriculture. Lead is a main constituent of several materials like ancient water pipes, lead-acid batteries, television screens and many others. Leaded gasoline was a major source originating lead salts. Other sources are solid waste combustion and industrial processes. The larger particles fall into the ground polluting soils or surface waters. The smaller ones travel long distances and remain in the atmosphere until it rains. Lead can end up in water and soils through corrosion of leaded pipelines and leaded paints. It cannot be broken down, only converted in other forms.	Lead accumulates in the body of water and soil organisms which suffer severe health effects from poisoning. Shellfish experience health effects at very small concentrations. Phytoplankton is an essential source of oxygen production and many larger sea-animals feed on it. If their body functions are disturbed global balances are negatively affected. Soil organisms and consequently soil functions are also affected by lead poisoning especially those near highways and farmlands. Lead is a truly dangerous threat as it accumulates not only in individual organisms but also in entire food chains.	Lead can enter human body through various routes, such as uptake of contaminated food, water and air. Fruits, vegetables, meats, seafood, soft drinks and wine may contain large amounts of it. Drinking water can become contaminated through corrosion of pipes especially and if is slightly acidic. Public water treatment systems are now required to perform pH-adjustments in drinking water. Cigarette smoke also contains lead but in small amounts.	Some of the most relevant health effects in humans are the disruption of the biosynthesis of hemoglobin leading to anemia, high blood pressure, kidney and brain damage, miscarriages and subtle abortions, sperm damage which causes declining fertility in men, disruption of nervous system, reduced learning capacities and behavior disturbance in children. Fetuses can be severely affected by lead once it passes through the placenta causing serious damages to their nervous system and brain.
Mercury (Group 3 / 3rd) Methylmercury (2B / 120th)	Mercury can be found naturally in the environment but rarely occurs free. It can be found in metal form, as salts or organic compounds. It enters the environment from normal breakdown of minerals in rocks and soils through exposure to wind and water. Human activities highly increase mercury levels in the environment, namely in air through fossil fuel, mining and smelting. The application of agricultural	Microorganisms can convert mercury present in surface waters and soils into methyl mercury, which may be quickly absorbed by many organisms. Fishes absorb and accumulate great amounts of this compound which may result in the accumulation in food chains. Some of the effects on animals are kidney and intestines damage, stomach	Mercury is not usually found in food but it can enter food chains trough smaller organisms that bioaccumulate it, and which are consumed by man. Mercury can enter the human body through the consumption of plants to which mercury-containing sprays had been applied. It is used in several household products such as	High exposure to mercury vapors causes harmful effects, such as brain and kidney damage, lung and eye irritation, skin rashes, vomiting and diarrhea. Other effects of mercury exposure are disruption of nervous system, DNA and chromosomal damage, negative reproductive effects like sperm damage, birth defects and miscarriages.

	fertilizers and the disposal of industrial wastewater releases mercury into soils and water. It is extremely used in thermometers and barometers (nowadays banned in the EU), recovery of gold from ores and more. Acid surface waters contain much higher mercury concentrations as it is mobilized from the ground.	disruption, reproductive failure and DNA alteration.	barometers, thermometers and fluorescent light bulbs. If some of those broke, high exposure can happen through breathing while it vaporizes.	
Nickel (Group 1 / 57th)	Nickel occurs in the environment at very low concentrations. It usually occurs in ores which are mined in various countries worldwide. It is applied as a component of steal and other products, including jewelry. Nickel is released into the air by power plants and trash incinerators. It settles to the ground or falls down with rain. Nickel can also end up in surface waters when present in wastewater streams. Most nickel released in the environment becomes immobile as it is absorbed in sediment or soil particles. In acid soils it becomes more mobile and often runs off to groundwater.	High concentrations of nickel on sandy soils can severely damage plants and on surface waters it can reduce the growth rates of algae. Microorganisms are also affected but they generally become quickly resistant to nickel. Nickel is an essential element for animals at low concentrations. It is extremely harmful when the maximum tolerable amount is exceeded as it can cause different kinds of cancer, especially in those organisms living near refineries.	Humans can be exposed to nickel by breathing contaminated air, drinking contaminated water, or eating contaminated food. Dermal exposure can also occur with contaminated soils and waters. Foodstuffs usually have small amounts of nickel. Chocolate and fats are an exception as they contain higher quantities. Smokers have high uptake of nickel through cigarette smoke.	Nickel is an essential element in small amounts but when the uptake is too high it can cause severe damages to human health. Sensitive individuals may develop dermatitis known as "nickel itch" after exposure to nickel and its compounds. Nickel exposure through breathing can cause pneumonitis as nickel fumes are respiratory irritants. Some of the most important effects are increased chances of developing lung, nose, larynx and prostate cancer, lung embolism, respiratory failure, birth defects, asthma and chronic bronchitis, and heart disorders.

* IARC classification according to IARC Monographs, Volumes 1–106 - (http://monographs.iarc.fr/ENG/Classification/ClassificationsGroupOrder.pdf) and ASTDR classification according to the ATSDR 2011 substance priority list - (http://www.atsdr.cdc.gov/SPL/index.html)

4.2 Toxicokinetics and bioaccumulation

Toxicokinetics involves the conversion of the external dose of a chemical to an internal dose leading to elimination from the body. In other words, it refers to the absorption, distribution, metabolism, and elimination of toxicants. Potential bioaccumulation in tissues and access and effects on the target organ/s depends on these processes. Absorption controls the uptake of metal(loid)s into the organism. The main routes are usually oral and inhalation, being dermal contact possible but at a very limited level (Dorne et al., 2011). They are then distributed to various areas depending on their properties and the different affinities for different cells and biomolecules. Blood and plasma are the main routes, particularly bonded to erythrocytes. A number of metal(loid)s may accumulate to a stable-state level in different body compartments (e.g., tissues, organelles) (McGeer et al., 2004).

For the majority of metal(loid)s the metabolic pathways are generally complex and multiples, and not always identified. Some metal(loid)s can undergo limited metabolism, either by conjugation or by eliminating a bound substance (e.g. As and its compounds). There is no degradation of the metal(loid) atom itself, but it may bind to a large variety of molecules in the organism (McGeer et al., 2004). Metal(loids)s can bind to biomolecules that are essential to cellular function (e.g., enzymes), alter their function, and cause toxicity.

Elimination occurs at a higher extent through urine (via kidney), and also to a much lower level in the gastrointestinal tract. The time needed for half of the initial amount of the metal(loid) to be excreted from the body (half-life) is highly variable and depends on the element (e.g., 10 to 12 years for Cd and Pb, 4 days for As, and 60 days for Hg) (Dorne et al., 2011).

Toxicokinetically, if the net-balance of uptake exceeds elimination for a metal(loid), then bioaccumulation occurs, such as when a metal has a high affinity for tissues that can act as a deposit (Weiss et al., 1996; WHO, 1995). For most metals, long-term accumulation occurs to a great extent in the kidney (e. g., As, Cd, and Hg) and blood (e.g., Pb).

It is important to notice that organisms have evolved in the presence of metal(loid)s and in many cases have developed appropriate strategies of their metabolism when concentrations exceed those normally encountered. For instance many metal(loid)s become associated with sulfur-rich proteins, particularly Class B ones (e.g., Ag and Hg) (McGeer et al., 2004).

One important factor that needs to be taken into account is the high inter-individual variability in human susceptibility due to genetic polymorphisms in the enzymes involved

in the metabolism of metal(loid)s. This is of major importance in the case of As (e.g., polymorphisms of arsenic-methyltransferases and glutathione-S-transferases omega 1 and 2) (Dorne et al., 2011).

Another key factor is the interactions among toxic and essential metals. Absorption, distribution, metabolism and elimination should be considered highly correlated for exposed individuals, with susceptibilities resulting in differential effects of multiple metal(loid)s (e.g., Fe inhibits Pb and Cd intestinal uptake due to shared absorption mechanisms; Se may potentially alter both As and methylmercury toxicity) (Sasso et al., 2010).

Concentration of metal(loid)s in human tissues and fluids is influenced not only by the environmental/occupational contamination, but also by diet, sex and age, although these factors influence elemental concentration to a lesser degree (Duyff, 2006). The use of cosmetics such as dyes and medical treatments can also influence the metal(loid)s concentration (Kanias, 1985).

4.3 Health effects

Most metal(loid)s are very toxic to living organisms and even those considered as essential can be toxic when in excess. They can disturb important biochemical processes, constituting an important threat for the human health. Major health effects include development retardation, endocrine disruption, kidney damage, immunological and neurologic effects, and several types of cancer (Mudgal et al., 2010). Health effects greatly depend on the element/mixture of elements subjects are exposed to. Health effects of the most toxic elements are summarized in **Table 1**.

4.3.1 Genotoxicity, immunotoxicity, and carcinogenicity

Genotoxicity can be referred as the ability to interact with DNA and/or the cellular apparatus that regulates the fidelity of the genome. These alterations can be caused either by direct interaction of metal(loid)s with nuclear DNA, or indirectly through reactive intermediates generated by the interaction of these elements with other cellular components, or both. When the inflicted damage cannot be repaired the cells undergo one of three following fates: necrosis (uncontrolled cell death), apoptosis (controlled cell death), or mutations, resulting from genetic code alterations fixed in the process of DNA duplication and transmission to daughter cells (Bal et al., 2011).

Several studies have been published about the genotoxic effects of metal(loid)s, demonstrating that elements like As, Cd, Cr, Fe, Hg, Mn, Ni, and Pb and their compounds are clastogens inducing micronucleus (MN), chromosomal aberrations (CA), sister chromatid exchanges (SCE) and aneugens inducing chromosomal loss (Jadhav et al., 2006).

Hartwig et al. (2004) postulated that some metal(loid)s may interfere with the fidelity of DNA repair, thus increasing genotoxic effects. Subsequent studies confirmed that metal(loid)s such as As, Cd, Co, and Ni interfere with both base and nucleotide excision repair (BER and NER) pathways (HERAG5, 2007).

There is considerable evidence that reactive oxygen species (ROS)-mediated oxidative damage induced by several metal(loid)s is the main pathway of their genotoxicity, particularly the ones proved to be carcinogenic (Henkler et al., 2010). Metal(loid)-induced oxidative stress has been shown to cause DNA damage through the production of three main species: superoxide anion radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\bullet$) (Jadvah et al., 2006).

A continuous disturbance of redox homeostasis can be associated with chronic pro-inflammatory signaling, leading to induction of proto-oncogenes and/or anti-apoptotic factors (Henkler et al., 2010). This causes a persisting overstimulation of the immune system, thus leading to immunotoxicity.

Immunotoxicity can be defined as any adverse effect on the structure or function of the immune system, or on other systems as a result of immune system dysfunction (Blank et al., 2000). The susceptibility of the immune system to exposure to metal(loid)s is well-known. Still reported effects in human populations for the majority of the elements are conflicting. The final outcome greatly depends on the element, its concentration, route of exposure, duration of exposure and biologic availability (Lehmann et al., 2011).

Certain metal(loid)s have been demonstrated to cause cancer in a variety of animal species. Based on epidemiologic data some of these metal(loid)s have been classified by IARC as human carcinogens (Group 1), namely As, Be, Cd, Cr, and Ni, and probably human carcinogens (Group 2B) – Co, Pb. Some of these elements have also been ranked in the top positions on a list of hazard substances by ATSDR in 2011 – **Table 2**.

Table 2. The ATSDR 2011 substance priority list. Adapted from http://www.atsdr.cdc.gov

2011 RANK	SUBSTANCE NAME	TOTAL POINTS	2007 RANK	CAS RN
1	ARSENIC	1665.5	1	007440-38-2
2	LEAD	1529.1	2	007439-92-1
7	CADMIUM	1318.7	7	007440-43-9
17	CHROMIUM, HEXAVALENT	1146.6	18	018540-29-9
57	NICKEL	998.7	53	007440-02-0

List of substances that are most commonly found at facilities on the National Priorities List (NPL) and which are determined to pose the most significant potential threat to human health due to their known or suspected toxicity and potential for human exposure at these NPL sites.

Regardless of their well recognized carcinogenicity, the mechanisms to which they transform cells are not yet understood, partially because of the complex nature of metal(loid)s interactions in biological systems. Many of them, including the carcinogenic ones, follow the metabolic pathways of similar essential metals. This is probably the result of similar binding preferences between metal(loid)s and nutritionally essential metals (Goyer, 2004).

Carcinogenic metal(loid)s are usually weak mutagens (except Cr), and they do not form DNA-adducts. The main pathway seems to be the elevated production of ROS they induce in the organism (Salnikow and Zhitkovich, 2008).

5. BIOMARKERS: HUMAN HEALTH

In order to better protect human health a process of population study known as molecular epidemiology has been developed to integrate laboratory measurements with epidemiological methodologies, linking individual exposure to an important biological event (Coelho et al., 2011a). The biological component is evaluated with biological markers, also called biomarkers, in different biological matrices, such as blood, urine, nails, hair, milk, and saliva, which are observable endpoints that indicate early events in the process leading up to disease (Gil and Pla, 2001). This is termed biological monitoring or biomonitoring and its main purpose is to relate the biomarker concentration to the internal dose, and then investigate the possibility to associate these data towards the effect or back to the source of the exposure (Needham et al., 2007).

In 2001 the World Health Organization (WHO, 2001) defined biomarker as a chemical, it's metabolite, or the product of an interaction between a chemical and some target molecule or cell that is measured in the human body. They commonly include biochemical,

molecular, genetic, immunologic, or physiologic signals of events in biologic systems (Schulte and Perera, 1993). The events are represented as a continuum between an external exposure to an agent and the resulting clinical effects (Albertini et al., 2006) (**Figure 2**). Exposure to these agents can happen through contact with contaminated air, water, soil and food, and also in the occupational environment and lifestyle factors. All these routes contribute to a complex exposure situation in daily life.
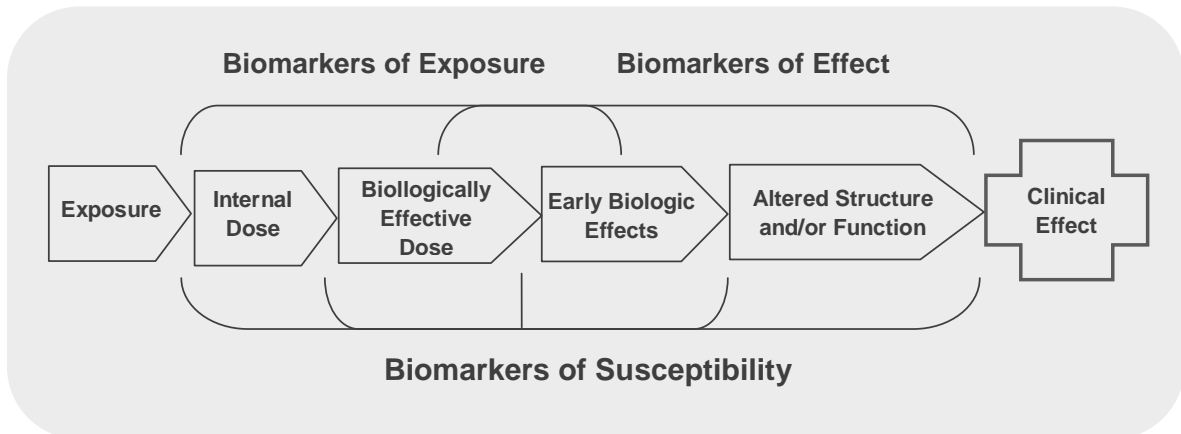
FIGURE 2 . Simplified diagram of the three categories of biomarkers along with the biological events taking place between exposure and clinical effect.

There are different classes of biomarkers. Traditionally these are classified as biomarkers of exposure, effect and susceptibility (**Figure 2**). Each of which is used to answer different questions and their accurate interpretation depends on the knowledge of the various transformations occurring in the metabolic pathways of the human body.

In 2006 the Committee on Human Biomonitoring for Environmental Toxicants of the National Research Council (NRC, 2006) as defined the three categories as:

- Biomarkers of exposure: a chemical, its metabolite, or the product of an interaction between a chemical and some target molecule or cell that is measured in the human body.

- Biomarkers of effect: a measurable biochemical, physiologic, behavioral, or other alteration in an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease.

- Biomarker of susceptibility: an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific chemical substance.

In order to achieve the mechanistic understanding of the biologic effect, and therefore a better prediction of disease risk, appropriate biomarkers of each category should be used. Examples of the most commonly used biomarkers are presented in **Table 3**.

TABLE 3. Examples of the most used biomarkers in each (sub)category. Based on the published literature review performed by Au et al. (2007).

Biomarkers of Exposure		Biomarkers of Effect	
Internal Dose	**Biologically Effective Dose**	**Early Biologic Effect**	**Altered Structure and/or Function**
Parent Compound/Metabolites	DNA/Protein Adducts		
		Reporter Gene Mutation	
		Altered Gene Expression	
		DNA Strand Breaks	
		Micronuclei	
		Chromosomal Aberrations	
			Cancer Gene Mutation
Biomarkers of Susceptibility			
DNA Sequence Variations			

5.1 Biomarkers of exposure

Biomarkers of effect can be divided in two subcategories: internal dose and biologically effective dose.

5.1.1 Biomarkers of internal dose

Biomarkers of internal dose aim to determine the compound or its metabolites in tissues or body fluids such as blood, urine, breast milk, and saliva. They can also give information on other sources of exposure to that compound and the existence of genetic polymorphisms for metabolic enzymes (Farmer et al., 2007).

Information on the elemental content of human samples, such as liver, kidney, brain, blood, and urine is well established (Mehra and Juneja, 2005). Obtaining nail and hair samples require a much less invasive option and provide a number of advantages, such as: ease of collection, storage and transportation; possibility to monitor elements accumulated over a period of a few weeks to a few months; long-term sample stability; and straight-forward analysis using conventional elemental analysis methods such as inductively coupled plasma-mass spectrometry (ICP-MS) and inductively coupled plasma-optical emission spectrometry (ICP-OES) (He et al., 2011). These techniques are the

most commonly performed and they have become routinely used for multi-elemental analysis of biological matrices (Caroli et al., 1992; Delves, 1988; Taylor et al., 2011).

For more than three decades, hair and nail samples have been used to assess environmental and occupational exposure to metal(loid)s (Bencjo, 1995; Bortoli et al., 1992; Brima et al., 2006; Button et al., 2009; Chatt et al., 1980; Creason et al., 1982; Kuangfei et al., 1999; Nowak and Kozlowski, 1998; Orloff et al, 2009; Rodushkin and Axelsson, 2000). As suggested by Rodushkin and Axelsson (2000) human hair and nails are also important biomarkers used for trace element analysis in order to identify diseases such as diabetes mellitus, coronary artery disease and other cardiovascular diseases. Also, mental retardation may be a result of Cd concentration.

Hair and nail structures incorporate elements in proportion to their dietary intakes and other exposures by various mechanisms including protein synthesis and chemical binding with sulfhydryl groups. They are useful markers for trace elements and are employed in clinical studies with increasing frequency. Hair was recommended as key sample to assess heavy metal concentration, according to the World Health Organization (WHO) (Druyan et al., 1998). In addition, the Environmental Protection Agency (EPA) and the International Atomic Energy Agency (IAEA) also suggest the use of human hair for environmental monitoring (Morton et al., 2002).

In cases where the environmental and occupational exposure to toxic elements needs to be assessed, nails are preferred to hair, as they don't easily be contaminated (Button, et al. 2009). It is also suggested that toenails may be a more reliable sample than fingernails, as they are less prone to environmental/occupational contamination (He, 2011). Fingernails come into contact with the atmospheric polluted air, metallic objects and other substances containing trace elements such as dyes. In contrast, toenails are hidden in the shoes and therefore they don't come into contact with many trace elements.

5.1.2 Biomarkers of biologically effective dose

Biomarkers of biologically effective dose assess the interaction of compounds with molecular targets such as DNA and protein receptors (e.g. measurement of DNA and protein adducts in urine and serum) (Amorim, 2003). Despite the presence of these adducts being readily measured, DNA adducts have become more popular and one of the most important biomarkers of exposure as their presence may be indicative of the risk associated with the exposure (Bonassi and Au, 2002).

Although biomarkers of exposure are highly relevant and specific indicators of an exposure, the information given does not necessarily translate into prediction of health consequence, and therefore other biomarkers need to be analyzed – biomarkers of effect and susceptibility (Au, 2007).

5.2 Biomarkers of effect

Biomarkers of effect can be divided in two subcategories: early biological effects and altered structure and/or function.

Biomarkers of early biological effects have improved accuracy for exposure assessment by providing objective measures on potential health effects at the level of the individual. They include several markers (**Table 3**) such as reporter gene mutation (e.g., *HPRT*, *HLA*, *GPA*, and *TCR* gene mutation assays), altered gene expression (e.g., expression from metabolizing genes, DNA repair genes, and specific enzymes), DNA strand breaks (quantified by the Comet Assay), cytogenetic markers such as micronuclei (MN) and chromosome aberrations (CA). From all the early biological effect markers CA assay is the most widely used and best validated biomarker (Bonassi and Au, 2002). The mechanisms are better understood and most environmental toxic substances have been shown to induce them (Au, 2007). As CA are also markers of altered structure and/or function they are extremely useful in cancer risk assessment. Most cancer cells and developmental abnormalities present these alterations (Au, 2007). Along with CA cancer gene mutation such as tumor suppressor genes and oncogenes are predictive markers for cancer morbidity and mortality.

5.2.1 T-cell receptor mutation assay

The T-cell receptor (TCR) is a heterodimer composed of two chains, α and β, that is expressed on the cell surface of the vast majority of peripheral T lymphocytes CD4^{+} and CD8^{+}. The genes coding for the two chains are located on chromosomes 14 and 7 respectively, and are phenotypically hemizygous. It is believed that only one of the two alleles is expressed actively as a result of allelic exclusion mechanism similar to that observed in the genes of immunoglobulins on B cells. It is therefore expected that a single mutation in the functional gene of the TCR results in the absence of phenotypic expression of the TCR on the cell surface (Kronenberg et al., 1986). In addition, the TCR can only be expressed on the cell surface in complex with CD3. The TCR and CD3 play an important role in antigen recognition and signal transduction so that the junction of the

two components is critical to these functions (Clevers et al., 1988). If the expression of some of chains of the TCR (α or β) is inactivated, the TCR complex α β / CD3 cannot be transported to the surface of the cell membrane and accumulates altered complexes in the cytoplasm (Akiyama et al., 1995).

Mutations in any of the alleles of the TCR may be detected by flow cytometry using antibodies that recognize the CD3 molecule. This technique allows to identify and enumerate the α β TCR mutants in the population of helper T cells expressing CD4 (Akiyama et al., 1995). It measures the frequency of mutant cells $CD4^+CD3^-$ among normal $CD4^+$ T cells. This method considers the total number of mutations in the TCR chain genes, being unable to differentiate between mutations occurring in the α chain gene and occurred in the β chain gene.

The TCR mutation assay is a simple and quick method for evaluating the mutagenic effects in human populations, requiring only a small volume of peripheral blood. Somatic genetic changes are important in cancer development because almost all cancer cells show genomic instability (Balmain et al., 2003). This instability may be induced by inherited mutations in genes, or mutations that are acquired in somatic cells during tumor development (Balmain et al., 2003).

The TCR mutation assay was shown to be a sensitive indicator for exposure to several polluting agents including radiation and chemicals (Kubota et al., 2000; Taooka et al., 2006), and determines the genotoxicity associated with exposures experienced in the period of time ranging from several months to 2-3 years (Taooka et al., 2006).

5.2.2 Micronuclei assay

Micronuclei (MN) are small nucleus formed whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division. Lagging whole chromosomes are due to defects in the chromosome segregation apparatus (e.g failure of the mitotic spindle, kitochore) or by damage to chromosomal substructures, mechanical disruption and hypomethylation of centromeric DNA (Albertini et al., 2000; Fenech et al., 2005). Chromosomal fragments may result from direct double-strand DNA breakage, conversion of single-strand breaks into double-strand breaks after cell replication, or inhibition of DNA synthesis (Mateuca and Kirsch-Volders, 2006).

The MN test in cultured peripheral blood lymphocytes is a well-established cytogenetic technique that has been extensively used as a biomarker of genotoxic exposure and early biological effects in human biomonitoring studies (Bonnasi et al., 2007; Carrano and

Natarajan, 1988; Fenech, 1993; Lando et al., 1998). This cytogenetic biomarker constitutes a valuable tool for studying environmental and occupational hazards to public health (Bonassi et al., 2005). It has been reported that this test is also predictive of cancer risk in human populations (Bonassi et al., 2007).

In the last two decades international efforts, such as the Human Micronucleus project (http://www.humn.org), greatly contributed to improvements in the reliability of the assay, providing guidelines on scoring criteria and analyzing major sources of variability (Kirsch-Volders et al., 2006). Compared with other cytogenetic assays, quantification of micronuclei confers several advantages, including speed and ease of analysis, and no requirement for metaphase cells (El-Zein et al., 2011).

MN can be scored in lymphocytes, in erythrocites and also in exfoliated epithelial cells from buccal or nasal mucosa, or urine. The former one is the standard *in vitro* MN test.

The development of the cytokinesis-block micronucleus (CBMN) assay, by addition of the actin polymerisation inhibitor cytochalasin B during the targeted mitosis, allows the identification of cells that have undergone one division as binucleated (**Figure 3**). This prevents confounding effects caused by differences in cell division kinetics since expression of micronuclei, nucleoplasmic bridges, or nuclear buds is dependent on completion of nuclear division (Fenech, 2000).

One of the major advantages of the CBMN assay is that in addition to measuring clastogenic effects easily, the aneugenic effects can also be detected (Martinez et al. 2004). Using fluorescent *in situ* hybridization (FISH) with probes targeted to the centromere region, one can determine if a whole chromosome or only a fragment was lost.
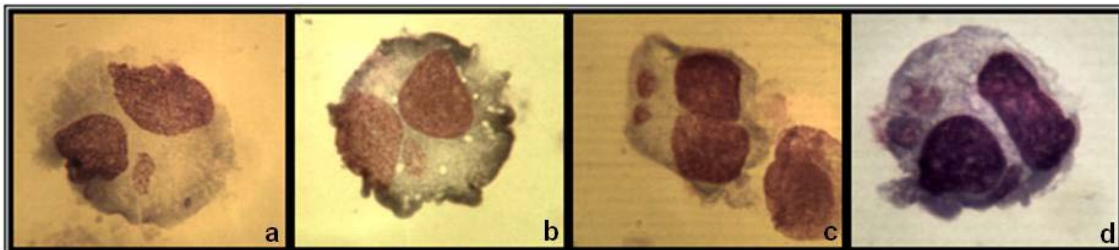
FIGURE 3. Micronuclei observed in binuclear lymphocytes from populations environmentally and occupationally exposed to metal(loid)s – Panasqueira mine area (1000x).
a) and **b)** binucleated cells with one micronuclei; **c)** binucleated cell with two micronucleus; **d)** binucleated cell with three micronucleus.

5.2.3 Chromosomal aberrations

Chromosomal aberrations (CA) are structural and/or numeric changes in the chromosomes. Structural changes are the result of chromosomal breaks (clastogenic effect) and the rearrangement within the chromosome or between chromosomes (**Figure 4**) (Delft et al., 1998). Numerical changes (i.e. aneuploidy, polyploidy) occur as a result of abnormal chromosomal segregation, either spontaneously or due to an aneugen treatment (Mateuca et al. 2006).

Visible structural changes in arrested metaphase-stage cells are usually divided in two types: chromosome-type aberrations (CA-chromosome) and chromatid-type aberrations (CA-chromatid). The former includes changes involving the two chromatids of one or more chromosomes while CA-chromatid includes changes involving only one of the two chromatids of one or more chromosomes (Mateuca and Kirsch-Volders, 2007).

In order for these two types of CA to happen one or more DNA double-strand breaks needs to take place. Their mechanism of formation seems to be different concerning the mutagen and also involve different mechanisms of DNA repair (Hagmar et al., 2004). CA-chromosome reflect incomplete or unrepaired double-strand breaks by the non-homologous end-joining and non-conservative homologous recombination repair mechanisms in G_0-G_1 phase. CA-chromatid reflect single-strand breaks and base modifications occurring essentially in S-phase.

Structural CA in lymphocytes have been used for more than 30 years in occupational and environmental settings as a biomarker of early effects of genotoxic carcinogens (Mateuca and Kirch-Volders, 2007). CA are indicative of potential risk of cancer, since they are associated with several types of human cancer. For example, many aberrations, particularly translocations and inversions are associated with morphological and phenotypic subtypes of leukemias, lymphomas and sarcomas (Heng et al., 2004).

CA assay is the most common technique to detect structural changes. Nevertheless this method is labor intensive and requires specifically skilled and experienced staff. More recent techniques using FISH methods allow an accurate and easy detection of structural CA but are significantly more expensive.
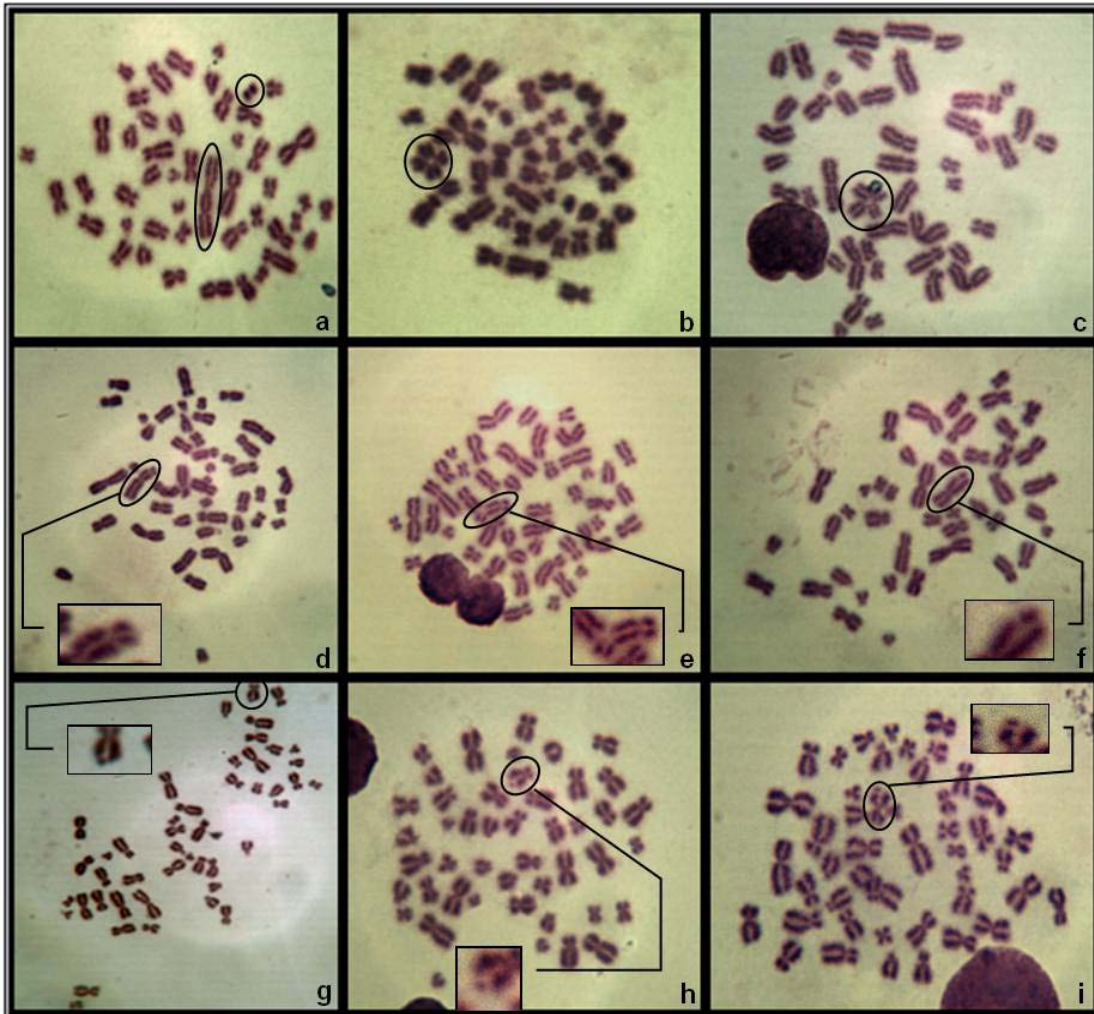
FIGURE 4. Chromosomal aberrations observed in metaphase lymphocytes from populations environmentally and occupationally exposed to metal(loid)s – Panasqueira mine area (1000x).
a) dicentric chromosome with accompanying fragment; **b)** and **c)** symmetrical tetraradial figures; **d)** gap in both chromatids; **e)** break in both chromatids; **f) - h)** break in one chromatid; **i)** gap in one chromatid.

5.2.4 Comet Assay

Comet assay (or single-cell gel electrophoresis - SCGE) is a simple, rapid and sensitive technique to assess genotoxic damage in single cells, using a small number of cells, therefore presenting advantages in comparison with other tests for genotoxicity (Tice et al., 2000). Studies with biomonitoring cytogenetic techniques use only proliferating cells and lymphocytes, while the comet assay can be applied to the proliferating cells, non-proliferative tissues and cells that are first to come into contact with mutagenic/carcinogenic substances (e.g. cells from oral and nasal mucosa) (Kassie et al., 2000).

The name Comet Assay is due to the fact that, after its completion, cells with damaged DNA present a comet shape, with an intensely bright head and a tail (**Figure 5**). The length of the tail and the brightness level is related to the number of breaks in the DNA chain. Cells without DNA damage are presented as intact nuclei without a tail.
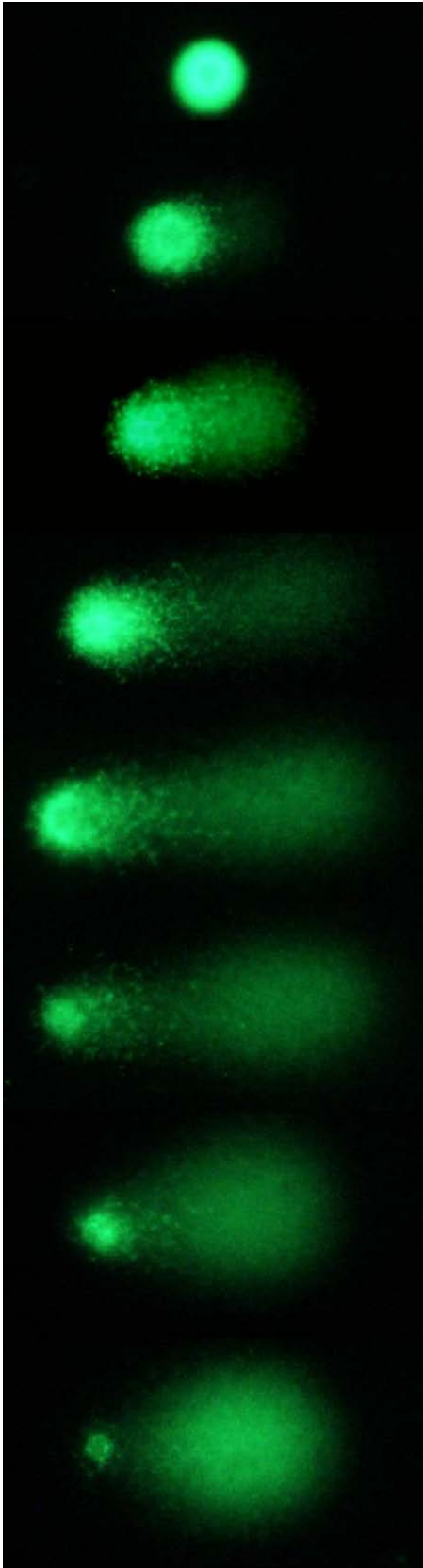
Since 1990 comet assay has been widely used in human biomonitoring studies. The sensitivity in detecting DNA damage in single cells is extremely important since the harmful effects of xenobiotics are specific to each cell and for each tissue (Singh et al., 1988).

The comet assay was introduced by Östling and Johanson in 1984. Briefly cells were embedded in agarose, placed on slides, then dipped in lysis solution (containing detergent and high salt concentrations), subjected to electrophoresis under neutral conditions and finally stained with acridine orange. It was developed to detect specifically DNA double-strand breaks.

This method was later modified by Singh and co-authors (1988), in which the assay is carried out under alkaline conditions (pH> 13). This led to a more sensitive version of this assay allowing the detection of both DNA double- and single-strand breaks, alkali labile sites, cross-links, and incomplete DNA repair sites. Nowadays this is the most frequently used method among published studies.

FIGURE 5 – Images of comets with different degree of damage (lymphocytes from populations environmentally and occupationally exposed to metal(loid)s – Panasqueira mine area).

Another procedure was developed by Olive and co-workers, which involves treatment of the slides with a alkaline lysis solution followed by electrophoresis at neutral pH conditions (Olive et al., 1990a) or mild alkaline, pH = 12.3 (Olive et al., 1990b) in order to detect single breaks in DNA.

Comets are observed in a fluorescence microscope after staining with suitable dye. The most common are ethidium bromide, propidium iodide, SYBR Gold and SYBR Green. Fluorescence intensity of the tail is proportional to the number of breaks in the DNA and it can be determined by direct observation under a microscope or using a computer program for analysis of images which provide a variety of parameters for each analyzed comet such as tail length, percentage of DNA in the tail (tail intensity), and tail moment (Kassie et al., 2000).

In recent years the comet assay has undergone several changes but the basic principles are based on neutral and alkaline versions. Due to its simplicity it can also be used in studies of DNA repair (Collins, 2004). These studies evaluated the ability of any cell to repair different types of damage in the DNA, including double and single breaks, base damage and cross-links.

It was also modified by Collins and co-workers to include an incubation step with lesion-specific enzymes. This extra step increases the sensitivity and selectivity of the assay converting damaged bases to DNA breaks. Some of the most commonly used are formamidopyrimidine DNA glycosylase (FPG), hOGG1 and endonuclease III (EndoIII).

The comet assay has several clinical applications, and it is widely used to evaluate the genotoxic potential of chemicals and environmental contaminants, and for environmental monitoring purposes (Kumaravel and Jha, 2006).

5.2.5 Immune markers

Biomarkers for the assessment of human immunotoxicity include the count of blood components, antibody-mediated immunity (serum concentrations of immunoglobulins), phenotype analysis of lymphocytes flow cytometry, among others (**Table 4**).

TABEL 4 – Biological markers of immunotoxicity in humans. Adapted from Gil and Pla (2001).

IMMUNE MARKER	EXAMPLES OF ENDPOINTS
Full blood count	Lymphocyte count
Study of antibody-mediated immunity	Immunoglobulin concentrations in serum: IgM, IgG, IgA, IgE
Phenotypic analysis of lymphocytes by flow cytometry	Surface markers: CD3, CD4, CD8, CD20, CD23, etc.
Study of cellular immunity	Delayed-type hypersensitivity on skin Natural immunity to blood group antigens: anti-A, anti-B
Auto antibodies & markers of inflammatory response	C-Reactive protein Autoantibodies to nuclei, DNA and mitochondria
Measure of non-specific immunity	Interleukine analysis: ELISA or RT-PCR Natural killer cell activity: CD56 or CD60 Phagocytosis: chemiluminescence Measurement of complement components

Lymphocytes are the primary cells involved in acquired immunity and their number may vary from 20 to 40% of the total cells in human blood. They are highly specialized cells that interact with other cells to initiate an immune response. The specificity of the receptor and functional heterogeneity allows them to respond to virtually any antigen (Descotes, 2004; Tryphonas et al., 2005).

T cells comprise approximately 50 to 70% of peripheral blood lymphocytes in humans and express in their surface the TCR, along with the cell differentiation markers CD2 and CD3. This type of cell can be divided into two subpopulations, $CD4^+$ and $CD8^+$, differentiated by their function and surface marker. T helper cells (Th) express on their surface marker CD4 and their main function is to assist B cells, by releasing cytokines, mainly by helping to produce antibodies in response to antigenic attack. This type of lymphocytes, in turn, is divided into two subtypes Th1 and Th2 cells that differ in the profile of cytokines they secrete. The Th1 subtype mainly secreted interleukin (IL) 2 (IL-2) and interferon γ (IFN-γ) and induces cellular responses that increase the microbicidal activity. The Th2 subtype secretes IL-4, IL-5 and IL-10, which stimulate B cells to produce antibodies (Descotes, 2004; Tryphonas et al., 2005). Cytotoxic T lymphocytes (Tc) express on their surface marker CD8 and their function is to capture the target cell through mechanisms of adhesion and release the contents of their granules into it. This induces apoptosis or disruption of the membrane and the death of the target cell (Abbas and Lichtman, 2004).

B lymphocytes are precursors of antibody-secreting plasma cells. They originate from hematopoietic stem cells located in the liver in the fetus, then later in the bone marrow,

and constitute approximately 10 to 20% of peripheral blood lymphocytes in humans. They can be identified by the presence of surface immunoglobulins - IgM, IgD, IgG, IgA or IgE - and various cell differentiation markers including CD19, CD22, CD23 and CD37. B lymphocytes are activated directly as a result of binding of the immunoglobulin expressed on the cell surface, an antigen or indirectly by interaction with T lymphocytes (Descotes, 2004; Tryphonas et al., 2005).

The main cells involved in nonspecific immune responses are natural killer (NK) and various phagocytic cells. NK cells are closely related to T cells, lacking their receptor and expressing CD16 and CD56 markers on their surface (Tryphonas et al., 2005). The main role of NK cells is to directly kill target cells by releasing cytotoxic mediators. NK cells can recognize and kill cells which are both covered with IgG, resulting in an antibody dependent cellular cytotoxicity, as well as cells expressing levels of antigens from the major histocompatibility complex (MHC) lower than normal (Descotes , 2004)

The importance of assessing changes in the percentages of different subpopulations is related to the existence of different studies linking exposure to certain substances with immunotoxic properties with these changes and their effect on the immune response (Biró et al., 2002; Boscolo et al., 1999, Oh et al., 2005; Tulinska et al., 2004). Hernandez-Castro et al. (2009) demonstrated that an increased, diminished, or absent function of immune-system cells is clearly associated with autoimmune diseases, deregulation of the immune response, and defective immune response against neoplastic cells or different pathogens. It is imperative to study homogeneous populations, as it is known that the number of lymphocytes vary depending on differing life situations (Moszczy´nski et al. 2001).

Each cell of the immune system has the ability to synthesize and release a variety of cytokines which travel to other cells (which can be immune or not) encouraging them to become more or less active. Any change in the function or the number of abnormal cells results in production of cytokines and a loss of control regulator (Tryphonas et al., 2005).

In the last decade pro-inflammatory cytokines have been related to cognitive decline and mood disorders. More recently Capuron et al. (2009) showed that increased concentrations of inflammatory markers were associated with reduced quality of life in elderly persons. These elements have the ability to influence the metabolism of neurotransmitters and neuroendocrine functions involved in the development of several behavior symptoms known as sickness behavior (Capuron et al., 2011). The metabolism of some of these neurotransmitters (e.g. serotonin, norepinephine, and dopamine) which

are synthesized within the brain from their precursors – tryptophan and tyrosine -, can be affected by immune activation.

Briefly, two enzymatic pathways can be activated: indoleamine-2,3-dioxygenase (IDO) pathway and the guanosine-triphosphatecyclohydrolase-1 (GTP-CH1) pathway.

When activated, IDO catalyzes the rate-limiting step of tryptophan conversion into kynurenine, being then degraded into several neuroactive metabolites, such as 3-hydroxykynurenine, quinolinic acid, and kynurenic acid (Chen and Guillemin, 2009). The kynurenine/tryptophan (kyn/trp) ratio reflects tryptophan breakdown and is considered to represent one estimate of IDO activity (Widner et al., 1997).

The activation of GTP-CH1 is responsible for the production of neopterin and tetrahydrobiopterin (BH4). Neopterin is released from human activated monocytes/macrophages and consequently allows to sensitively monitoring the degree of immune activation (Fuchs et al., 1992, 1993, 1997). BH4 is a cofactor of aromatic amino acid hydroxylases and also nitric oxide synthases (NOS) (Neurauter et al., 2008). The former ones contribute to the biosynthesis of monoamines and NOS in the conversion of arginine to nitric oxide (NO) (Neurauter et al., 2008). The estimation of NO production is usually evaluated by the quantification of the stable NO metabolite – nitrite (NO_2^-) (Capuron et al., 2011).

In humans, an increased formation of neopterin and enhanced degradation of tryptophan have been found in viral infections, malignant disorders and autoimmune diseases (Weiss et al., 1999). The activation of BH4 is believed to be associated with acute inflammatory processes, and the inhibitory role of neopterin with chronic inflammatory processes (Neurauter et al., 2008). Accelerated tryptophan degradation and consequently elevated kyn/trp ratio in serum/plasma has been shown to strongly correlate with neopterin concentrations, and in the vast majority of the studies, degradation of tryptophan was found to be associated with the extent, the activity and the course of the diseases (Jenny et al., 2011). In a context of chronic inflammation, lower levels of nitrite can reflect an imbalance in oxidant/antioxidant mechanisms with reduced antioxidant defenses or impairment in NOS activity (Capuron et al., 2011).

There is an increasing amount of information reporting immunotoxic effects of metal(loid)s in animals (Cabassi 2007). As for humans, such investigations are still lacking and most results are inconsistent.

5.3 Biomarkers of susceptibility

The expression of all the previously described biomarkers (exposure and effect) is significantly influenced by individual factors, acquired (e.g., life styles like smoking habits, and alcohol consumption) and genetic susceptibility categories (e.g., inheritance of variant genes that affect chemical metabolism and DNA repair, etc.) (Au, 2007).

It is well known and recognized that even under identical exposure conditions different individuals have different responses. Therefore some individuals are more susceptible/resistant to certain exposures than others. In order to identify these variations investigations with biomarkers of susceptibility have focused on DNA sequence variation in certain genes, such as the ones involved in chemical metabolism and DNA repair, genes related to immune function, and cell cycle control. These studies provide valuable information about the influence of such genes on specific effects of exposure(s) and response to genotoxic agents (Kyrtopoulos, 2006).

The study of genetic variability had a clear impact on the project of sequencing the human genome (Bernstein et al., 2012; Sachidanandam et al., 2001). Several types of polymorphisms in the human genome were identified and they include insertions or deletions of one or more bases, duplications, inversions, repeats and genomic rearrangements, but the vast majority (68%) consists of SNPs (Single Nucleotide Polymorphisms). SNPs are single base pair positions in genomic DNA where different sequence alternatives (alleles) exist in normal individuals in some populations, in which the least frequent allele has an abundance of 1% or greater (Brookes, 1999). They can be found in all genomic regions (exons, introns, promoter regions/regulatory and intergenic regions). This variety of locations makes them more susceptible to allow for functional or physiological allelic relevance compared to other types of polymorphisms .

In the beginning of 2000, most molecular epidemiological studies, aimed to identify SNPs associated with individual susceptibility to multifactorial disease, and were mainly focused on SNPs located in coding regions or regulatory regions of the genome since these changes are likely to result in phenotypic changes, given the strong possibility of affecting the function or expression of a particular protein (Feuk et al., 2006; Gaspar et al., 2006). Several SNPs have been identified in genes involved in codification of enzymes responsible for chemicals metabolism or cell response to DNA damage. These diverse forms of the same gene can lead to a difference in sensitivity of cell to chemicals effect (Norppa, 1997).

Polymorphic genes involved in the metabolism of contaminants in human body may modulate the levels of biomarkers arising from environmental and/or occupational

exposure to genotoxic agents (Pavanello and Clonfero, 2000). The identification of genetic polymorphisms which have a key role in the modulating genetic damage can help minimize risks for susceptible subjects (Costa et al., 2008).

5.3.1 Polymorphisms in genes involved in the metabolism (phase I and phase II)

Phase I of metabolism involves mainly oxidations, reductions and hydrolysis and works to introduce a polar group into the molecule (contaminant). In what concerns chemical metabolizing enzymes, those of human cytochrome P450 (CYP) are the most studied ones. They present polymorphisms that appear to influence observed damage. CYP enzymes can both detoxify or activate chemicals (Werck-Reichhart and Feyereisen, 2000).

Phase II of metabolism consists primarily on conjugation reactions that combine the products of phase I reactions with one of several endogenous molecules to form water-soluble products. Among phase II enzymes, glutathione S-transferases (GST) are the most important group of detoxifying enzymes, being responsible for the glutathione conjugation with reactive species of many chemicals. Based on amino acid similarities, seven classes of cytosolic GSTs are recognized in mammalian species designated Alpha (GSTA), Mu (GSTM), Pi (GSTP), Sigma (GSTS), Theta (GSTT), Omega (GSTO), and Zeta (GSTZ) (Andonova et al., 2010).

Polymorphisms of some of these enzymes (GSTT1, GSTM1 e GSTP1) have been associated with an increase of cancer risk (Bolognesi, 2003; Sundberg et al., 1998). In what concerns to alpha class GST (GSTA – GSTA1 e GSTA2) there are few epidemiological studies about the role of these enzymes in cancer susceptibility (Silva et al., 2009).

Specific details will only be given for studied polymorphic genes.

5.3.1.1 *GSTA2*

The alpha class of GSTs is greatly expressed in liver, kidney and adrenal tissue. It is one of the most versatile GST families as it is responsible for GSH conjugation of compounds such as bilirubin, bile acids and penicillin, thyroid and steroid hormones, allowing their solubilisation and storage in the liver (Tetlow and Board, 2004). GSTA1 and GSTA2 are the most expressed alpha GSTs enzymes in the liver.

Since the Alpha family is involved in a wide range of processes, such as steroid biosynthesis and providing protection against alkylating agents, polymorphic variations in these genes can be responsible for physiological consequences that can change the susceptibility to disease and chemical response (Silva et al., 2009). Up till now only a few epidemiological studies regarding the role of polymorphic alpha class GST in cancer susceptibility (Silva et al., 2009).

Two members of this class - GSTA1 and GSTA2 - catalyze the GSH conjugation of a large variety of electrophiles, possess glutathione-dependent steroid isomerase activity and glutathione-dependent peroxidase activity (Coles and Kadlubar, 2005). Particularly the *GSTA2* gene is thought to represent a main line of defense against oxidative stress (Tetlow et al., 2004).

Different polymorphisms in *GSTA2* gene were described by Tetlow et al. (2004) in three different ethnic populations - *P110S, S112T and E210A*, all of them being in strong linkage disequilibrium (Silva et al, 2009). The S112T polymorphism in exon 5 position 63 is the consequence of a base substitution (G -> C) that leads to change of the amino acid serine (Ser) to tryptophan (Trp). Gemignani and co-workers (2007) reported an association between *GSTA2* (S112T) variant allele and an increased risk of lung cancer.

5.3.1.2 *GSTM1*

The human *GSTM1* gene is located on the chromosome 1 and is polymorphic. Three alleles of *GSTM1* are commonly described - *GSTM1*A, GSTM1*B,* and *GSTM1*0* (Strange et al., 2000). *GSTM1*A* and *GSTM1*B* alleles differ by only one amino acid (Lys172Asn) and have no apparent differences in substrate specificity between them; *GSTM1* positive is defined as wild type (Parl, 2005). Conversely, *GSTM1**0 is a deleted allele, and the homozygous genotype (*GSTM1*-null) expresses no GSTM1 protein (Huang et al., 2009).

About 53% of Caucasians lack GSTM1 activity, but ethnic differences in gene frequency are evident in a meta-analysis published by Garte and Gaspari (2001); the frequency of *GSTM1*-null genotype is similar in Asians but lower in African-Americans (27%). *GSTM1* genotype is typically determined using a PCR-based assay that indicates the *GSTM1* null/positive genotype. However, this approach does not identify the null allele and, therefore cannot distinguish homozygous positive from heterozygous individuals (Parl, 2005). This differentiation can only be achieved by real time PCR genotyping.

5.3.1.3 *GSTP1*

The *GSTP1* gene is located on the chromosome 11 (Parl, 2005). Several single nucleotide polymorphisms have been described in this gene that yield an amino acid change in the encoded protein (Kitteringham et al., 2007). It was reported that codons 105 and 114 of GSTP1 are linkage disequilibrium between two loci (Harris at el., 1998). The polymorphism in the codon 105 (isoleucine -> valine) is the consequence of a base substitution (A -> G) that leads to an amino acid substitution. This amino acid substitution close to the GSTP1 substrate-binding site results in a significant reduction in the protein activity (Gaspar et al., 2004), and is one of the most widely studied to date.

The effect of the Val 105 allele appears to differ according to the substrate. Enzymes with Val105 have a 7-fold higher efficiency for PAH diol epoxides than the enzymes with Ile105. In contrast, the Val105 enzyme is 3-fold less effective using 1-chloro-2,4-dinitrobenzene (Strange et al., 2000).

About 50% of Caucasians present the Val 105 allele, but ethnic differences in gene frequency are evident in a meta-analysis published by Mo et al. (2009); the frequency is much lower in Asians (27%) and much higher in African-Americans (83%).

5.3.1.4 *GSTT1*

The gene encoding human glutathione S-transferase T1 (GSTT1) was localized in human chromosome 22 (Landi, 2000). A polymorphism for this enzyme was first characterized in 1994 (Pemble et al., 1994). It consists of a deletion of the gene, resulting in the lack of active *GSTT1* enzyme. The genotype with the homozygous deletion of the *GSTT1* gene is designated *GSTT1*-null, whereas the genotype having at least one copy of the gene is designated *GSTT1*-positive (Landi, 2000).

The frequency of null genotypes has been assessed in different countries; approximately 20% Caucasians present the null genotype while among Asians the frequency of null genotype is much higher (about 47%) (Landi, 2000; Garte and Gaspari, 2001). Similarly to what happens in *GSTM1* polymorphism determination, *GSTT1* genotype is also usually categorized as null or positive without identification of heterozygous individuals.

5.3.2 Polymorphisms in genes involved in DNA repair

DNA repair is a key human cellular response to DNA-damaging stimuli. DNA repair mechanisms are vital responses to multiple types of DNA damage, including the ones

caused by exposure to environmental and endogenous carcinogens (Kiyohara and Yoshimasu, 2007; McWilliams et al., 2008). Most of the alterations caused, if not repaired, can result in genetic instability, mutagenesis and cell death. Thus genetic variations in DNA repair genes may modulate DNA repair capacity and, therefore influence risk for cancer development (Kiyohara et al., 2007).

At present, two major mechanisms have been identified and characterized for DNA repair of single strand breaks: base excision repair (BER) and nucleotide excision repair (NER). The mismatch repair (MMR) pathway plays an essential role in the correction of replication errors such as base-base mismatches and insertion/deletion loops To repair double-strand breaks (DSB), cells mainly rely on non-homologous end joining (NHEJ) and homologous recombination (HR) (Christmann et al., 2003).

Briefly, BER, NER, and MMR pathways are excision repair processes that depend upon complementary DNA strands to direct their substitution of excised base(s). The BER pathway recognizes and removes incorrect or damaged bases using a family of DNA N-glycosylases that result in the formation of an apurinic/apyrimidinic (AP) site. There are two main types of BER mechanisms, one involves DNA polymerase beta-dependent single nucleotide repair and the other, long-patch pathway, requires proliferating cell nuclear antigen (PCNA). Whereas BER processes are dependent upon specific N-glycosylases to recognize mismatches or damage, the NER pathway recognizes abnormal structures and chemistry via heterodimers composed of DNA damage-binding protein 1 (DDB1), xeroderma pigmentosum complementation group E (XPE), xeroderma pigmentosum complementation group C (XPC) or HR23B proteins. Defect in NER has been associated with xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy (TTD) (Atain and Krebs, 2006). The MMR pathway repairs mismatches and loops generated by insertions or deletions. MMR of damaged bases is frequently mediated by the BER pathway.

HR employs one of two related but distinct mechanisms. Synthesis-dependent strand annealing (SDSA) requires RAD51, RAD52, RAD54, RAD55, RAD57 and RAD59 and also that a single DNA strand finds its complementary sequence within a double-stranded DNA. Single-strand annealing (SSA) only requires the association of two complementary strands and RAD52 and RAD59. NHEJ is homologous DNA template-independent and facilitates direct modification and ligation of two DNA ends present in DSB (Atain and Krebs, 2006).

Key DNA repair proteins/enzymes presenting polymorphic forms are expected to have great health impact. Some studies suggest their influence on observed genetic damage (Goode et al., 2002; Norppa, 2004) but no definite conclusions can be taken yet. In fact,

repair pathways are complex and influence of a single variant may be difficult to detect (Zijno et al., 2006).

In this study we analysed polymorphisms of enzymes involved in BER (*APEX1, MPG, MUTYH, OGG1, PARP1, PARP4*, and *XRCC1*) and NER (*ERCC1, ERCC4*, and *ERCC5*) pathways.

5.3.2.1 Polymorphisms in genes involved in BER pathway

5.3.2.1.1 *XRCC1*

The *XRCC1 (X-ray repair cross-complementing group I)* gene is located on chromosome 19 and encodes a protein involved in the DNA repair processes by base excision repair. This protein is non-enzymatic and facilitates protein-protein interactions to coordinate BER and single-strand break repair responses (Wilson et al., 2011). There is evidence of 21 SNPs in the *XRCC1* gene that produce amino acid substitutions, some of them occurring at sufficiently high frequency for association studies. Among these are two non-synonymous polymorphisms: one at codon 194 of exon 6 that causes a change of amino acid arginine (Arg) by tryptophan (Trp), and another one at codon 399 of exon 10 that causes a change of the amino acid arginine (Arg) to glycine (Gln). The Trp194 variant has been associated with increased BER capacity (Wilson et al., 2011) while the Gln399 variant has been associated with reduced repair capacity (Duell et al., 2000).

5.3.2.1.2 *APEX1*

The *APEX1 (apurinic/apyrimidinic endonuclease 1)* gene is located on chromosome 14 and encodes the major apurinic/apirimidinic endonuclease in mammalian cells, which plays a central role in the BER repair pathway. This enzyme hydrolyses the phosphodiester backbone immediately 5' to AP sites in DNA. These AP sites are cytotoxic and mutagenic lesions that can alter normal DNA replication if not repaired (Cao et al., 2011). In addition, this gene also plays an important role in redox regulation of transcription factors, helping controlling cellular response to oxidative stress. It is also involved in cytokine expression and cell death and participates in the processes of class switch recombination during the production of antibodies (Guikema et al., 2007; Tell et al., 2009). From the 18 polymorphisms described in this gene, one of the most frequent is located in exon 5, codon 148 and involves a change of the amino acid asparagine (Asp) to

glutamic acid (Glu) in the synthesized protein. It is situated in the region between those required for redox activity and AP (Xanthoudakis et al., 1994).

5.3.2.1.3 *MPG*

The *MPG (N-methylpurine-DNA glycosylase)* gene is located on chromosome 16 and encodes a protein responsible for the removal of N-alkyl purines (mainly, 3-alkyl adenine) induced in DNA by simple monofunctional alkylating agents (Bessho et al., 1993). One of the most studied polymorphisms is located at codon 22 of exon 3 and involves a change of the amino acid lysine (Lys) to glutamine (Gln), which lowers the activity of the enzyme synthesized (García-Lestón, 2012a).

5.3.2.1.4 *MUTYH*

The *MUTYH (mutY homologue)* gene is located on chromosome 1, and encodes a DNA glycosylase which cuts adenine bases in the DNA in places where it is incorrectly matched with guanine, cytosine or 8-oxoGua (Wilson et al., 2011). In human cells multiple forms of the protein MUTYH are express due to multiple sites of transcription initiation and alternative splicing of mRNA transcripts (Shinmura et al., 2000). One of the multiple polymorphisms that has been studied is located in codon 335 of exon 12 and causes a change of the amino acid glutamine (Gln) to histidine (His), in the synthesized protein.

5.3.2.1.5 *OGG1*

The *OGG1 (8-oxoguanine glycosylase)* gene is located on chromosome 3, and encodes a protein responsible for excision of 8-oxoGua, the main base injury caused by oxidative damage to DNA. If this damage is not cleaved, it can pair with adenine leading to a GC to TA transversion. Therefore, a decrease in the activity of OGG1 may lead to mutations that activate oncogenes or inactivate tumor suppressor genes (Laantri et al., 2011, Roberts et al., 2011). The most common polymorphism in this gene is located at codon 326 of exon 7 and causes a change of the amino acid serine (Ser) to cysteine (Cys), resulting in a protein which exhibits a capacity slightly defective, which can contribute to their inability to coordinate with other proteins in the BER repair pathway (Goode et al., 2002).

5.3.2.1.6 *PARP1*

The *PARP1 [poly (ADP-ribose) polymerase family, member 1]* gene is located on chromosome 1 and it encodes a chromatin-associated enzyme, poly (ADP-ribosyl) transferase, which modifies several nuclear proteins by ribosylation. The modification is dependent on DNA and it acts by unwinding chromatin thus promoting cell cycle progression and the recruitment of various repair proteins (Malanga and Althaus, 2005). One of the most studied polymorphisms is located in codon 762 of exon 17, and involves the change of the amino acid valine (Val) to alanine (Ala) in the synthesized protein. This polymorphism is found in the catalytic domain, influencing the activity of the enzyme kinetics (Kauppinen et al. 2006).

5.3.2.1.7 *PARP4*

The *PARP4 [poly(ADP-ribose)polymerase family, member 4]* gene is located on chromosome 13 and encodes a protein similar to the one just described, which is capable of catalyze ribosylation reactions. This protein has a catalytic domain that is homologous to the poly (ADP-ribosyl) transferase, but lacks the zinc finger domain necessary to bind to the N-terminal of DNA. Since this protein is not capable of binding directly to DNA, its transferase activity could be activated by protein-protein interactions mediated by other regions of the protein (Still et al., 1999). Two of the multiple polymorphisms that have been studied are located in codon 1280 and 1328 of exon 31, which cause a change of the amino acid gycine (Gly) to arginine (Arg) and of Proline (Pro) to threonine (Thr), respectively.

5.3.2.2 Polymorphisms in genes involved in NER pathway

5.3.2.2.1 *ERCC* family

The excision repair cross-complementing (*ERCC*) gene family is involved in the reduction of damage to DNA by NER. Modified nucleotides along with adjacent nucleotides are removed from the damaged strand during the first step (excision), which is followed by synthesis of an intact strand through DNA polymerase activity (repair synthesis) (Hoeijmakers, 1993).

The *ERCC1* gene encodes a protein that along with ERCC4 function in a complex involved in the 5' incision made during NER (van Vuuren et al., 1995). This complex is a

structure specific DNA repair endonuclease that interacts with essential meiotic endonuclease 1 (EME1). The *ERCC5* gene (xeroderma pigmentosum, complementation group G - XPG) encodes a protein that is involved in excision repair of UV-induced DNA damage.

Mutations in the *ERCC1* gene result in cerebro-oculo-facio-skeletal (COFS) syndrome (Suzumura and Arisaka, 2010) and polymorphisms that alter expression of this gene may play a role in carcinogenesis (Goode et al., 2002). Defects in *ERCC4* gene are a cause of xeroderma pigmentosum complementation group F (XPF), or xeroderma pigmentosum VI (XP6). Mutations in the *ERCC5* gene cause Cockayne syndrome, which is characterized by severe growth defects, mental retardation, and cachexia (Cleaver et al., 1999).

Some of the most studied polymorphisms of this family are ones located in codon 504 of 3′-untranslated region of ERCC1 and causes a change of the amino acid lysine (Lys) to Glutamine (Gln); in codon 415 of exon 8 of ERCC4 and causes a change of the amino acid arginine (Arg) to Glutamine (Gln); in codon 1104 of exon 15 and in codon 529 of exon 8 of ERCC5 and causes a change of the amino acid aspartic acid (Asp) to histidine (His) and cysteine (Cys) to serine (Ser), respectively.

It is widely known that mining activities are one of the most hazardous both in the occupational and the environmental context. The lack of reliable information on health impacts related to the contamination of the Panasqueira mine area draw attention to the need of a community and workers health study. All the available information in the literature was careful analysed and the project was designed and executed accordingly.

II. AIM OF THE STUDY

Few studies were conducted in our country on the effects of mining activities in human populations and there is little information about these conditions.

Environmental studies performed in Panasqueira mine area identified an anomalous concentration of several metal(loid)s in stream sediments, superficial and ground waters from local courses, road dust, soils, and plants for human consumption from nearby villages. In the absence of information of the health impacts of the environmental and occupational exposure to this contamination, we considered of major importance the development of an appropriate population-based study, with the objective/aim of characterizing the state of health of the communities affected, in particular the potential geno- and immunotoxic risk of that environment. This work, as previously mentioned is part of a program of actions defined in in the National Action Plan for Environment and Health (PNAAS – *Plano Nacional de Acção Ambiente em Saúde*): Action I.7 - survey of human health effects associated with pollutants in soils and sedimentary materials and definition of intervention strategy.

There is a further need for a deeper and long-term evaluation of the mining impacts on workers and communities' health. Appropriate environmental laws with adequate monitoring enforcement need to be adopted to prevent most of the damage caused in mine communities. All these measures will help to better protect the health and safety of people working in, living near and those otherwise impacted by historic, current and proposed mines.

The results of this project will have a major impact on the Central region of Portugal. Nevertheless, the results and methodology developed during the project to address this important issue will be useful for populations worldwide, particularly communities living in the vicinity of mines and working in them. Results can be very useful to epidemiologists, physicians and for institutions involved in regulatory affairs, especially for better document awareness raising campaigns.

Considering all the points previously mentioned the main objective of this study was to evaluate the effect of the external contamination on selected indexes of internal dose from subjects environmentally and occupationally exposed, relate this to the genotoxic and immunotoxic damage and also evaluate the possible modulating role of genetic polymorphisms involved in metabolism and DNA repair.

A multiple approach was used in order to integrate all studied biomarkers: exposure, effect and susceptibility, which will enable a better characterization of the risk. Concentrations of several elements in blood, hair, fingernail and toenail samples, quantified by ICP-MS (As, Cd, Cr, Hg, Mn, Mo, Ni, Pb, and Se), or ICP-OES (Ca, Cu, Fe, K, Mg, Na, S, Si and Zn), were

used as biomarkers of internal dose. Genetic damage was studied by means of cytogenetic tests (MN and CA), a somatic cell mutation assay (TCR mutation assay) and Comet Assay. Percentages of different lymphocyte subsets and concentrations of neopterin, tryptophan, kynurenine, and nitrite were selected as immunotoxicity biomarkers. For all these exposure and effect biomarkers, the role of potentially confounding factors, such as age, gender and life style factors, was also evaluated. Finally, genetic polymorphisms in genes involved in the metabolism (*GSTA2*, *GSTM1*, *GSTP1*, and *GSTT1*) and in the DNA repair (*XRCC1*, *APEX1*, *MPG*, *MUTYH*, *OGG1*, *PARP1*, *PARP4*, *ERCC1*, *ERCC4*, and *ERCC5*) were investigated as biomarkers of susceptibility.

III. MATERIAL AND METHODS

1. STUDY POPULATION

1.1 Population Selection

Study population was chosen according to previous publications with environmental data from the Panasqueira Mine area, particularly the reports from the National Institute of Engineering, Technology and Inovation (INETI - Instituto Nacional de Engenharia, Tecnologia e Inovação) published within the scope of e-Ecorisk programme mention in the review of the literature. Chosen villages were S. Francisco de Assis and Barroca do Zêzere as exposed and Casegas and Unhais-o-Velho as control.

In order to establish a protocol of participation and cooperation of health services of the areas covered in this study, we contacted the Department of Public Health and Planning from the Regional Health Administration, Centre region (ARSC, IP – Administração Regional de Saúde do Centro). This protocol was established (see **annex I**) and meetings with the local authorities of each village were scheduled.

Campaigns to sensitize populations to participate in this study were carried out along with the local health services, local authorities and the priests of the villages. Objectives, importance and other relevant information from our study was provided in a leaflet (see **annex II**). This leaflet also contained the address, telephone number and email to clarify any doubt.

These campaigns were fairly successful in some of the villages as many people showed great interest in participating, and we had a high level of participation in the administration of the health questionnaires and the sample collection stage.

Study subjects were contacted by local authorities who provide them a sterile bottle for first morning urine collection and two bags for finger and toenails collection. Sample collection and administration of the health questionnaires took place on Sunday mornings.

All subjects were fully informed about the procedures and objectives of this study and each of them signed an informed consent prior to the study (see **annexe III**). Ethical approval for this study was obtained from the institutional Ethical Board of the Portuguese National Institute of Health.

1.2 Study group

The study population consisted of a total of 122 subjects living in the area of the Panasqueira mine. Forty-one individuals living in villages located in the vicinity of the mine and downstream the Zêzere river (S. Francisco Assis and Barroca do Zêzere – **Figure 6**) were classified as environmentally exposed (16 males and 25 females), 41 male miners and ex-miners from the Panasqueira mine represented the group of occupationally exposed, and 40 additional subjects without environmental and/or occupational exposure to mining activities, or other known toxic exposure, were the controls. This latter group included individuals living in non contaminated areas upstream the river and on the western side of the mine (Casegas and Unhais-o-Velho – **Figure 6**). Control individuals worked mainly in administrative offices and were matched with the environmentally exposed group by age, gender, lifestyle, and smoking habits (17 males and 23 females). Only individuals aged over 18 years and living in the same village for at least 5 years before the study were selected (see **annex IV**).
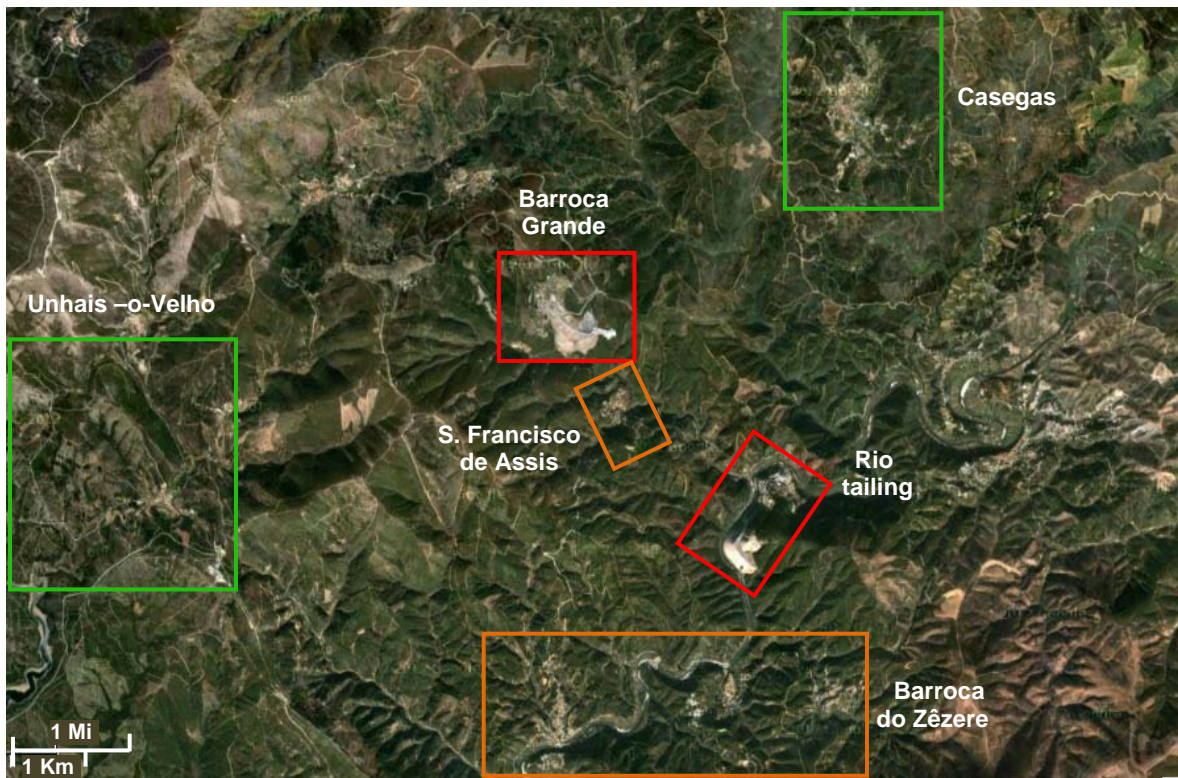
FIGURE 6 – Map showing the location of the 4 study villages: the 2 exposed ones are highlighted with orange squares (S. Francisco de Assis and Barroca do Zêzere), and the 2 controls in green squares (Casegas and Unhais-o-Velho). In the red squares are the main sources of contamination: active tailing (Barroca Grande) and old one (Rio tailing).

Health conditions, medical history, medication, diagnostic tests (X-rays, etc.), and lifestyle factors were assessed by a questionnaire (see **annexes V & VI**). Subjects also provided information about the presence of specific symptoms related to metal(loid)s exposure and chronic respiratory diseases, such as bronchitis and others; drinking and agricultural water source; agricultural practices, including pesticides usage; diet. The general characteristics of the study groups are summarized in **Table 5.**

Occupationally exposed individuals were also inquired about the years of work and how long ago they stop working. The group was composed of 7 current miners and 34 ex-miners. From the current miners group 3 were from S. Francisco de Assis (exposed village) and 4 from Unhais-o-Velho (control). As for the ex-miners group 14 were from S. Francisco de Assis and Barroca do Zêzere (exposed villages) and 20 from Unhais-o-Velho (control).

Possible differences between groups regarding several variables were assessed. No significant differences in age were observed between the three groups (p=0.063). Smoking habits groups were established as never/ever smokers, since the number of ex-smokers was extremely high and the majority of these individuals had been heavy smokers. Gender and smoking habits difference (p<0.001 and p=0.001, respectively) among groups were mostly due to the fact that the occupationally exposed group was composed only of males, the vast majority of whom were smokers. Significant differences were found also for the frequency of individuals reporting their involvement in agricultural practice (p=0.001), while no significant differences were found for the source of drinking water (p=0.714), quantity of fish consumed (p=0.541), and pesticide use in the last year (p=0.408).

Table 5. Characteristics of the study population: baseline comparison between controls and exposed groups by demographics and lifestyle factors.

	Controls	Environmentally Exposed	Occupationally Exposed	*P-value*
Total	40	41	41	
Gender				
Males	17 (43%)	16 (39%)	41 (100%)	**<0.001**[b]
Females	23 (59%)	25 (61%)	0 (0%)	
Age (years)[a]	56.60 ± 12.58	61.71 ± 13.50	62.05 ± 7.57	0.063[c]
Smoking habits				
Never smokers	25 (62%)	32 (78%)	16 (39%)	**0.001**[b]
Ever smokers	15 (38%)	9 (22%)	25 (61%)	
Years working (current miners)	-	-	25.07 ± 7.07	
Ex-miners			34	
Years Working	-	-	18.31 ± 10.19	
Years Stop Working	-	-	16.25 ± 18.13	
Village				
S. Francisco de Assis	0 (0%)	19 (46%)	9 (22%)	**<0.001**[b]
Casegas	16 (40%)	0 (0%)	0 (0%)	
Barroca do Zêzere	0 (0%)	22 (54%)	8 (19%)	
Unhais-o-Velho	24 (60%)	0 (0%)	24 (59%)	
Water consumption				
Bottled water	2 (5%)	3 (7%)	4 (10%)	0.714[b]
Tap water	20 (51%)	23 (56%)	17 (41%)	
Spring water	17 (44%)	15 (37%)	20 (49%)	
Fish consumption				
0-2 portions/week	19 (47%)	25 (61%)	23 (56%)	0.541[b]
>2 portions/week	20 (53%)	16 (39%)	18 (44%)	
Agriculture				
No	6 (15%)	14 (34%)	1 (2%)	**0.001**[b]
Yes	34 (85%)	27 (66%)	40 (98%)	
Pesticide usage (last year)				
No	10 (26%)	16 (39%)	11 (28%)	0.408[b]
Yes	28 (74%)	25 (61%)	29 (72%)	

[a]Mean±standard deviation; [b]Chi-square test; [c]ANOVA test

1.3 Sample Collection

Blood samples were collected by venipuncture in:

- BD Vacutainer® CPT™ cell preparation tubes with sodium heparin for mononuclear leukocyte isolation prior to TCR mutation assay and comet assay;

- tubes containing sodium heparin for cytokinesis block MN test and chromosomal aberrations, and for genetic polymorphisms genotyping;

- tubes containing ethylenediamine tetraacetic acid (EDTA) for metal(loid)s quantification, analysis of lymphocytes subsets, and quantification of neopterin, tryptophan, kynurenine and nitrite.

First morning urine samples (minimum volume: 20mL) were collected in polyethylene sterile bottles.

Hair and nail (finger and toe) samples were collected with stainless steel scissors and nail clippers and stored in polyethylene bags.

Samples were transported under refrigeration and kept at approximately +4ºC (blood for TCR mutation assay, comet assay, cytokinesis block MN test and chromosomal aberrations) and -20ºC (blood for genetic polymorphisms genotyping and metal(loid) quantification; plasma for quantification of neopterin, tryptophan, kynurenine and nitrite; urine, hair and nails) until analysis. All samples were coded and analysed under blind conditions.

2. BIOMARKERS OF EXPOSURE

2.1 Metal(loid)s in blood, urine, nail and hair samples

2.1.1 Instrumentation

The inductively coupled plasma-mass spectrometry (ICP-MS) instruments used were a PerkinElmer Elan DRC II, and a Thermo Elemental X Series. A Perkin-Elmer Optima 5300 and a Thermo Scientific iCAP 6500 were used for inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis. For microwave digestion an Anton Paar Multiwave 3000 oven, equipped with 16 vessels was used. Before use and between each batch of samples, the polyether ether ketone (PEEK) utensils were thoroughly washed with acid and then rinsed with deionised water.

2.1.2 Reagents and standards

All reagents used were trace analysis grade or better quality. All aqueous solutions were prepared using ultra-pure water (>18Ω cm^{-1}). For analysis of blood samples In and Sc were the chemical elements used as internal standards while Ge and Ir were used for urine, hair and nail samples analysis.

2.1.3 Sample preparation

Blood samples (1-2g) were added to 2mL of HNO_3 in Teflon vials and digested for 24h at 100°C. After cooling, the digested solutions were diluted with deionised water up to 10mL in polypropylene tubes for elemental analysis by ICP-MS and ICP-OES.

Urine samples were defrosted 24h hours before the analysis, centrifuged for 3min at 2500rpm and diluted up to 25 fold with 1% v/v HNO_3 for elemental analysis by ICP-MS and ICP-OES.

Fingernail and toenail samples were washed thoroughly following a slightly modified version of the protocol described by Slotnick et al. (2007). Visible exogenous material was first removed using plastic forceps. Samples were then placed in clean glass vials and sonicated for 5min using acetone, rinsed with deionised water followed by acetone, sonicated for 10min with deionised water, then rinsed twice with deionised water, ensuring complete submersion of the sample during each step. All samples were oven dried at 60ºC overnight.

Hair samples were washed following a slightly modified version of the protocol described by Schrauzer et al. (1992); this involved washing the samples four times with 1% Triton X-100, rinsing once with acetone, three times with deionised water, and twice more with acetone. Each sample was washed for approximately 30min (total time) and subsequently all the samples were dried in an oven at 70°C for 2h.

Hair, finger and toenail samples were acid digested using closed vessel microwave assisted heating. Into each vessel 2mL of HNO_3 was added to accurately weighed samples (200mg when enough sample) and left to stand for 1h before sealing the vessels. The microwave heating program was: 100% power (1200W), 15 min ramp to 170ºC, held for 10min, then held for 20min at room temperature. The pressure in the system was approximately 200psi under these conditions. This method resulted in complete sample dissolution. The solutions were transferred to plastic tubes and made up to 5mL with ultra-pure water for direct determination via ICP-MS and ICP-OES.

2.1.4 Quality control and quality assurance

Blood analyses were validated using European Union, Institute for Reference Materials and Measurements - Certified Reference Materials (CRMs) BCR 634, BCR 635, and BCR 636. Recoveries varied between 81 and 83% and the precision of the method (for Cd and Pb) was better than 10% coefficient of variation.

Freeze-dried human urine CRM from National Institute of Environmental Studies (NIES), Japan, was used in addition to validating the metal quantification in urine samples, this material was also analysed during each analytical run as a quality control (QC) sample. Results from CRMs were within the reference range for As, Pb and Se.

As no nail CRMs were available, the methods for digestion and analysis of hair, fingernail and toenail samples were validated using a human hair CRM - NCS DC 73347a and NCS ZC 81002b human hair (NCS Beijing, China). The CRMs were also used as QC standard by digesting a portion with each batch of samples and determining the metal concentration along with the other digests. Results from CRMs were within the reference range. Other QC measures used in the different matrices included the periodic analysis of suitable standards to check on instrument drift and short-term stability.

2.1.5 Determination of creatinine

Creatinine adjustment is routinely used in urine analyses to reduce some factors that are not related to metal(loid) exposure, such as urine concentration and urine volume (Hinwood et al., 2002). Results of urine samples were adjusted and they are reported as µg/g creatinine.

Creatinine was measured photometrically using the Jaffe reaction (Roche Diagnostics).

3. BIOMARKERS OF EFFECT

3.1 T-cell receptor mutation assay

Isolation of mononuclear leukocytes was performed using BD Vacutainer® CPT™ cell preparation tube with sodium heparin, following manufacturer's instructions. The mononuclear leukocytes layer (buffy coat) was removed and washed three times with ice-cold phosphate buffer solution (PBS) pH 7.4, at 1000rpm (~270×g) for 10 min.

T- cell receptor (TCR) mutation assay was carried out using a flow cytometric method following the protocol described by García-Lestón et al. (2011). In brief, mononuclear cells were stained with 7-amino-actinomycin D to gate out dead cells, and with fluorescein-isothiocyanate (FITC)-labeled anti-CD3 and phycoerytrin (PE)-labeled anti-CD4 antibodies (Becton Dickinson). Cell suspensions were analyzed by a FACScalibur flow cytometer with *Cell Quest Pro* software (Becton Dickinson). A minimum of 2.5×10^5 lymphocyte-gated events were acquired, and mutation frequencies of TCR (TCR-Mf) were calculated as the number of events in the mutant cell window ($CD3^-CD4^+$ cells) divided by the total number of events corresponding to $CD4^+$ cells.

3.2 Cytokinesis Block MN Assay

Aliquots of 0.5 mL of heparinised whole blood were used to establish duplicate lymphocyte cultures for cytokinesis-blocked micronucleus test, as described in Costa et al. (2006). Cultures were incubated at 37ºC in the dark for a total of 72h, and cytochalasin B (6 µg/mL) was added at 44h to prevent cytokinesis. Cells were collected by centrifugation and treated twice with a mixture of RPMI (pH 7.2) supplemented with 2% fetal bovine serum. The cells were centrifuged again and submitted to a mild hypotonic treatment in a mixture of RPMI (pH 7.2):deionised water (1:4,v/v), supplemented with 2% fetal bovine serum. Then, the centrifuged cells were placed on dry slides and smears were performed. After air-drying, the slides were fixed with cold methanol:acetic acid (3:1, v/v). Air-dried slides were stained with 4% Giemsa in pH 6.8 phosphate buffer.

Microscopic analyses were performed on a Vickers Instruments light microscope. To determine the total number of MN in binucleated cells, a total of 1000 binucleated cells with well-preserved cytoplasm (500 per replicate) were scored for each subject, using a 400X magnification for detection and a 1000X magnification for confirmation. MN were scored blindly by the same reader and identified according to the criteria defined by Fenech (2007).

3.3 Chromosomal aberrations, aneuploidies and gaps

Aliquots of 0.5mL of heparinised whole blood were used to establish duplicate lymphocyte cultures the chromosomal aberrations assay as described in Roma-Torres et al. (2006). Cultures were incubated at 37ºC in the dark for 48h. Democolcemid (0.11µg/mL) was added and the culture was continued for a further period of 3h. Cells were harvested by centrifugation, subjected to hypotonic treatment (KCl 0.075mol/L, 37ºC for 10min), fixed twice with freshly prepared cold methanol:acetic acid (3:1), placed on slides and stained with 4%Giemsa in phosphate buffer pH6.8 for 10min.

Microscope analyses were performed on a Vickers Instruments light microscope and scored blind by the same reader. One hundred metaphases were analysed for each individual, fifty from each culture duplicate, using a 1000X magnification according to the criteria of Therman et al. (1980).

 Cells with 46 chromosomes were scored for structural aberrations, according to:

- total CA frequency was defined as the number of aberrations, excluding gaps;

- chromosome-type aberrations (CA-chromosome) included chromosome-type breaks, ring chromosomes, and dicentrics;

- chromatid-type aberrations (CA-chromatid) included chromatid-type breaks.

Aneuploidies (An) – cells with 45 and 47 chromosomes – and gaps (single and double) were also scored.

3.4 Comet assay

Peripheral blood mononuclear leukocytes were isolated as described in the previous section (TCR mutation assay protocol). Cells were suspended in freezing medium (50% foetal serum, 40% RPMI 1640, 10% DMSO) to obtain 10^7cells/mL, and stored at -80ºC until time of analysis. On the day of the analysis cells were quickly thawed at 37ºC and cell viability was accessed by trypan blue exclusion technique being in all cases higher than 85%.

The alkaline version of the comet assay was performed as described by Costa et al. (2008) with minor modifications. Briefly, cells collected by centrifugation (9000rpm for 3min), and suspended in 100µL of 0.6% low-melting-point agarose in PBS (pH 7.4), and dropped (5µL drops) onto a frosted slide precoated with a layer of 1% normal melting point agarose (4 drops per individual, 12 drops per slide). Slides were placed in the fridge for 10 min and allowed to solidify. Slides were then immersed in freshly prepared lysing

solution (2.5M NaCl, 100mM Na$_2$EDTA, 10mM TrisBase, 0.25M NaOH, pH 10 supplemented with 1% triton X-100 30min before use) for 1h at 4ºC, in the dark (fridge). After lysis, slides were placed on a horizontal electrophoresis tank in an ice bath. The tank was filled with freshly made alkaline electrophoresis solution (1mM Na$_2$EDTA, 300mM NaOH, pH 13) to cover the slides, and they were left for 20min in the dark to allow DNA unwinding and alkali-labile site expression. Electrophoresis was carried out for 20min at 30V and 300mA (1 V/cm). The slides were then washed for 10min with PBS 7.4 followed by 10min wash with ice-cold bidistilled water. Slides were then dehydrated by placing them for 15 min in 70% and 96% ethanol solutions.

Prior to analysis, gels were stained with 120µL of SYBR Green solution (1µL of SYBR green 10000X + 10mL of TE, aliquoted per 1mL and stored at -20ºC). After staining the slides were washed twice with ice-cold bidistilled water and let to dry for 30min to 1h. Before scoring a drop of water and a cover slip were placed on the top of each slide.

A 'blind' scorer examined 25 randomly selected cells from each gel (100 cells/donor) using a magnification of 400X. Image analysis was performed with Comet Assay IV software (Perceptive Instruments). Comet tail length (TL), percentage of DNA in the tail (tail intensity - %TDNA) and tail moment (TM) were the DNA damage parameters evaluated. For the statistical analysis only %TDNA was analysed according to what has been recommended by Kumaravel et al. (2009).

3.5 Analysis of lymphocytes subsets

The percentages of different lymphocyte subsets, namely T lymphocytes (CD3$^+$ lymphocytes), T helper (Th) lymphocytes (CD4$^+$ lymphocytes), T cytotoxic (Tc) lymphocytes (CD8$^+$ lymphocytes), B lymphocytes (CD19$^+$ lymphocytes), and natural killer (NK) cells (CD16$^+$ and CD56$^+$ lymphocytes) were evaluated by flow cytometry as described in García-Lestón et al. (2011).

Three-color direct immunofluorescence surface marker analysis was performed to determine the percentages of the following lymphocyte subpopulations: T lymphocytes, T helper (Th) lymphocytes, T cytotoxic (Tc) lymphocytes, B lymphocytes and natural killer (NK) cells. Whole blood collected in EDTA containers (100µl) was incubated for 15min in the dark with the following antibodies (Becton Dickinson), according to manufacturer's instructions: FITC-labeled antiCD3 (for T lymphocytes), PE-labeled antiCD4 (for Th lymphocytes), phycoerytrin-cyanin 5 (PE-Cy5)-labeled antiCD8 (for Tc lymphocytes), PE-Cy5-labeled antiCD19 (for B lymphocytes), and PE-labeled CD16 and CD56 (for NK cells). The erythrocytes were removed by lysis through the addition of FACS Lysing

solution (Becton Dickinson). After washing with PBS (phosphate buffer solution), cells were fixed with CellFix (Becton Dickinson), and analysed within 24h on a FACScalibur flow cytometer using *Cell Quest Pro* software (Becton Dickinson). After gating the lymphocytes based on forward/side scatter plots, fluorescence data of FL1 (FITC), FL2 (PE) and FL3 (PE-Cy5) were obtained. At least 10^4 events in the lymphocytes window were acquired.

3.6 Quantification of neopterin, tryptophan, kynurenine, and nitrite

Neopterin concentration was determined by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (BRAHMS, Hennigsdorf, Germany), according to the manufacturer's instructions. The limit of detection (LOD) was 2 nmol/L neopterin.

A high-performance liquid chromatography (HPLC) methodology with 3-nitro-L-tyrosine as internal standard was used as previously described (Widner et al., 1997) to measure tryptophan and kynurenine concentrations. The kynurenine to tryptophan ratio (Kyn/Trp) was calculated to estimate the extent of tryptophan breakdown and expressed in micromoles kynurenine per millimole tryptophan.

In order to estimate nitrite acid (NO) production, the stable NO metabolite nitrite (NO^{2-}) was determined by the Griess reaction assay (Promega, Madison, Wisconsin) (Griess, 1879).

4. BIOMARKERS OF SUSCEPTIBILITY

4.1 DNA extraction

Genomic DNA was obtained from 200 µL of heparinised whole blood samples using a commercially available kit according to the manufacturer's instructions (QIAamp DNA extraction kit - Qiagen, Hilden, Germany). Each DNA sample was stored at -20 ºC until analysis.

4.2 Genotyping of polymorphisms in gene involved in the metabolism

4.2.1 *GSTA*

The *GSTA2* Ser112Thr (rs2180314) polymorphisms was genotyped by real-time PCR (AB7300) using TaqMan SNP Genotyping Assays from Applied Biosystems (ABI Assays reference: C_22275149_30, respectively) according to the manufacturer's instructions. To carry out the allelic discrimination the DNA samples were quantified by PicoGreen dsDNA Quantification Reagent (Molecular Probes, Eugene, OR, USA) according to the manufacturer's recommendations

4.2.2 *GSTM1* and *GSTT1*

GSTM1 and *GSTT1* genotyping for gene deletions were carried out by a multiplex PCR as described by Lin et al. (1998) with minor modifications described in Teixeira et al. (2004). DNA samples were amplified with the primers: 5´-GAA CTC CCT GAA AAG CTA AAG C-3´and 5´-GTT GGG CTC AAA TAT ACG GTG G-3´ for GSTM1, which produced a 219bp product and 5´-TCA CCG GAT CAT GGC CAG CA-3´ and 5´-TTC CTT ACT GGT CCT CAC ATC TC-3´ for GSTT1, which produced a 459bp product. Amplification of albumin gene with the primers 5´-GCC CTC TGC TAA CAA GTC CTA C-3´ and 5´-GCC CTA AAA AGA AAA TCC CCA ATC-3´ was used as internal control, and produced a 350bp product. PCR was performed in a final volume of 50µL, consisting of DNA (0.1µg), dNTP (0.2mM each) (Perkin-Elmer), MgCl$_2$ (2.5mM), each primer (1.0, 0.3 and 0.2µM for GSTM1, GSTT1 and albumin, respectively), AmplitaqGold polymerase (1.25U) (Perkin-Elmer), reaction buffer and 2% DMSO. Amplification was performed with an initial denaturation at 95ºC for 12min, followed by 35 cycles of amplification with 94ºC for 1min, 62ºC for 1min and 72ºC for 1min, and a final extension step at 72ºC for 10min. A GeneAmp 9600

thermal cycler (Perkin-Elmer) was used. The amplification products were visualised in an ethidium bromide stained 1.5% agarose gel.

4.2.3 *GSTP1*

The *GSTP1* Ile105Val (rs1695) polymorphism was determined by PCR and RFLP according to the method of Harries et al. (1997),with minor modifications described in Teixeira et al. 2004. DNA samples were amplified with the primers: 5´-ACC CCA GGG CTC TAT GGG AA-3´ and 5´-TGA GGG CAC AAG AAG CCC CT-3´ (Perkin-Elmer). The PCR amplification was carried out with 50ng DNA in 10mM Tris–HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.3mM dNTP (Perkin-Elmer), 50ng of each primer and 1.25U of Taq polymerase (AmplitaqGold; Perkin-Elmer) in a total volume of 50µL. Amplification was performed with an initial denaturation at 95ºC for 7min, followed by 35 cycles at 94ºC for 30s, 62ºC for 30s, and 72ºC for 30s, and a final extension at 72ºC for 10min. The amplification product (20µL) was digested with five units of *Bsm*AI (New England Biolabs) in 50mM NaCl, 10mM Tris–HCl, 10mM, and 1mM dithiothreitol (DTT) at 55ºC incubation for 16h. The resulting fragments lengths were then separated on a 2% agarose gel and stained with ethidium bromide (0.5mg/mL). When the *Bsm*AI restriction site was present, the fragment of 176bp was digested into two fragments of 91 and 85bp. Homozygous wild type individuals (Ile/Ile) lacked the 91 and 85bp fragment, and heterozygous (Ile/Val) had three bands; homozygous individuals (Val/Val) lacked the large parent band and had the two smaller bands.

4.3. Genotyping of polymorphisms in genes involved in DNA repair

4.3.1 *XRCC1, APEX1*, *MPG, MUTYH, OGG1*, *PARP1* and *PARP4*

The *XRCC1* Arg194Trp (rs1799782) and Arg399Gln (rs25487), *APEX1* Asp148Glu (rs1130409), *MPG* Lys17Gln (rs3176383), *MUTYH* Gln335His (rs3219489), *OGG1* Ser326Cys (rs1052133), *PARP1* Val762Ala (rs1136410), and *PARP4* Gly1280Arg (rs13428) and Pro1328Thr (rs1050112), gene polymorphisms were determined by Real-Time PCR using TaqMan[®] SNP Genotyping Assays from Applied Biosystems (ABI Assays reference: C_11463404_10, C_622564_10, C_8921503_10, C_32323403_10, C_27504565_10, C_3095552_1, C_1515368_1, C_8700143_10, and C_8700142_10, respectively) following Conde et al. (2009), Gomes et al. (2010) and Silva et al. (2010). In order to carry out the allelic discrimination for these polymorphisms the DNA samples

were quantified using the Quant-iT™ Picogreen® dsDNA Assay Kit (Invitrogen) according to the manufacturer's recommendations.

The Real-Time PCR amplification was performed in 10µl reactions containing 10ng of genomic DNA, 1X SNP Genotyping Assay Mix (containing two primer/probe pairs in each reaction and two fluorescent dye detectors - FAM® and VIC®) and 1X TaqMan Universal PCR Master Mix containing the AmpliTaqGold® DNA polymerase, dNTPs and optimized buffer components. The amplification conditions consisted of an initial AmpliTaq Gold® activation at 95°C during 10min, followed by 40 or more amplification cycles consisting of denaturation at 92°C for 15s and annealing/extension at 60°C for 1min. Approximately 10-15% of the genotype determinations were carried out twice in independent experiments with 100% of concordance between experiments.

4.3.2 *ERCC1*, *ERCC4* and *ERCC5*

ERCC1 Lys504Gln (rs3212986), *ERCC4* Arg415Gln (rs1800067), and *ERCC5* Asp1104His (rs17655) and Cys529Ser (rs2227869) polymorphisms were determined using the TaqMan SNP genotyping assay (Applied Biosystems, codes C_2532948_10, C_3285104_10, C_1891743_10 and C_15956775_10, respectively), following Costa et al. (2008). The PCR amplification was performed in 10µL reactions containing 10ng of genomic DNA, 1X SNP Genotyping Assay Mix, and 1X TaqMan Universal PCR Master Mix containing optimised buffer components and Rox reference dye. The amplification conditions consisted of an initial AmpliTaq GoldR activation at 95ºC during 10min, followed by 40 or more amplification cycles consisting of denaturation at 92ºC for 15s and annealing/extension at 60ºC for 1min. Amplification was performed in the 7300 Real-Time PCR System (Applied Biosystems) and sequences were detected by the SDS-Sequence Detection Software (version 1.3.1). All the genotype determinations were carried out twice in independent experiments and all the inconclusive samples were reanalyzed.

5. STATISTICAL ANALYSIS

A general description of the study population was performed through univariate analysis. The distribution within the three study groups of gender, age and lifestyle factors potentially influencing the levels of studied biomarkers (i.e., smoking habits, water and fish consumption, agricultural activity and use of pesticides) was evaluated with the Chi-square test for categorical variables and the analysis of variance (ANOVA) for continuous variables.

The effect of exposure on the concentration of metals was preliminarily tested with the ANOVA of log-transformed data. A multiple regression analysis was performed to estimate the effect of the exposure, adjusted for actual confounders. All models included age and smoking habits. Three multiple regression models were applied for the multivariate analysis, depending on the characteristics and statistical distribution of variables. Details are given in **Table 6**. Associations between two variables were tested by Spearman correlation.

Table 6. Multiple regression models applied according to the characteristics and statistical distribution of variables.

Log-linear	Poisson	Negative-binominal
Fe-B, Mg-B, S-FN, S-TN, S-H, Se-B, Si-B	As-H, Cd-B, Cd-U, Cd-FN, Cd-TN, Cd-H, Cr-U, Cr-H, Hg-U, Hg-FN, Hg-TN, Hg-H, Mn-H, Ni-H, Pb-FN, Pb-TN, Se-FN, Se-TN, Se-H	As-B, As-U, As-FN, As-TN, Cr-FN, Cr-TN, Cu-B, Cu-H, Fe-H, Mg-FN, Mg-TN, Mg-H, Mn-B, Mn-U, Mn-FN, Mn-TN, Mo-B, Ni-U, Ni-FN, Ni-TN, Pb-B, Pb-U, Pb-H, Se-U, Zn-B, Zn-FN, Zn-TN, Zn-H

The effect of exposure on the level of genotoxicity biomarkers was preliminarily tested through ANOVA. To achieve a better approximation to the normal distribution, a log-transformation of the data was applied to TCR-Mf and %DNAT. No transformation was needed for MN frequency. The Kruskal-Wallis test was performed for CA-total, CA-chromosome, CA-chromatid, and aneuplodies. Best fitting multiple regression models were used to estimate the effect of the exposure. Linear regression was applied on the log-transformed TCR and %DNAT; negative binomial regression on non transformed data was carried out with MN and CA-total; lastly, Poisson regression on non transformed data was fitted for CA-chromosome, CA-chromatid, and aneuploidies. All models included age, smoking habit (as previously mentioned smokers were classified as ever/never smokers given the high number of heavy smokers that declared to be ex-smokers), and actual confounders. A possible role as effect modifiers of candidate biomarkers of susceptibility,

on the genotoxic damage induced by exposure, was also tested. When the number of subjects with homozygous mutations was small, these were merged with the group of subjects with heterozygous mutations. Thus, a dominant model was hypothesized in this case; an additive model was tested in all other cases. Mean ratio (MR) was used as the point estimate of effect accompanied by its 95% confidence interval (CI). To take into account the village of origin and to evaluate the accumulation of exposure from the work and the environment, the occupationally exposed population was divided into: i) subjects occupationally and environmentally exposed (working in the mine and living in villages near the mine); ii) subjects only occupationally exposed, (working in the mine and living in villages upstream the river). Linear regression on log-TCR-Mf and log-%DNAT, and negative binomial regression on CA-total were fitted to estimate the effect of exposure according to these new groups. Adjustment for age, smoking habit and parameter-specific actual confounders was applied. An ancillary analysis was carried out to quantitatively assess the association between metal concentration and genotoxicity. The study subjects were divided into three groups according to the tertile distribution of each metal. The resulting three-level factors (one factor for each metal) were, in turn, fitted in a regression model on the genotoxicity biomarkers. For each biomarker, the best fitting regression method was chosen. All models included age, smoking habits, and model-specific confounders.

The effect of exposure on the level of immunotoxicity was preliminarily tested with the ANOVA. To achieve a better approximation to the normal distribution, a log-transformation of the data was applied to neopterin, tryptophan, kynurenine and nitrite levels, kynurenine/tryptophan ratio and percentages of the different lymphocyte subsets. A multiple linear regression analysis was performed to estimate the effect of the exposure on the log-transformed data. Adjustment for age and smoking habits was applied. For all biomarkers, actual confounders were identified and estimations adjusted accordingly. A sub-analysis on the control and environmentally exposed population was performed to evaluate the role of gender as a confounder and/or effect modifier. An ancillary analysis was carried out to quantitatively assess the effect of metal concentration on biomarkers of immunotoxicity. The study subjects were divided into three groups according to the tertile distribution of each metal. The resulting three-level factors (one factor for each metal) were, in turn, fitted in a linear regression, to the log-transformed value of immunotoxicity biomarkers. All models included age, smoking habits, and model-specific confounders. A logistic regression model was applied to identify the relationship between selected symptoms and environmental and/or occupational exposure. Adjustment for age, smoking

habits and model-specific confounders was applied. Associations between two variables were tested by Spearman correlation.

The critical limit for significance was set at *P*<0.05. The statistical software used for the analyses were StataCorp. 2011, Stata Statistical Software: Release 12, College Station, TX: StataCorp LP, and SPSS Inc. Released 2004, SPSS for Windows, Version 13, Chicago, SPSS Inc.

IV. RESULTS

1. BIOMARKERS OF EXPOSURE

Results of metal(loid) quantification showed significant differences among groups, for several elements, in different matrices (**Table 7**).

TABLE 7. Concentrations of metal(loid)s in the different biological samples in the exposure groups (mean ± SD).

	Controls	Environmentally Exposed	Occupationally Exposed	Statistical significance	Published/ reference ranges[a]
As-B (µg/L)	11.67 ± 6.58	17.23 ± 11.13	14.71 ± 12.48		2.6-17.8
As-U (µg/g creat)	60.17 ± 72.28	93.34 ± 105.38	71.38 ± 118.07		2.3-161
As-FN (µg/g)	0.14 ± 0.10	0.61 ± 1.04	1.31 ± 2.88	**	0.07-1.09
As-TN (µg/g)	0.22 ± 0.30	0.65 ± 0.56	1.01 ± 2.36	**	0.07-1.09
As-H (µg/g)	0.12 ± 0.14	0.14 ± 0.15	0.32 ± 0.52	**	0.03-0.32
Ca-B (mg/L)	61.38 ± 6.77	60.89 ± 8.20	59.82 ± 6.87		49.5-62.3
Cd-B (µg/L)	2.44 ± 0.68	2.31 ± 0.32	2.62 ± 0.94		0.15-2.04[b]
Cd-U (µg/g creat)	0.47 ± 0.34	0.57 ± 0.45	0.47 ± 0.31		0.06-0.79
Cd-FN (µg/g)	0.12 ± 0.13	0.15 ± 0.22	0.10 ± 0.12		0.01-0.44
Cd-TN (µg/g)	0.04 ± 0.04	0.05 ± 0.04	0.07 ± 0.13		0.01-0.44
Cd-H (µg/g)	0.04 ± 0.05	0.06 ± 0.08	0.15 ± 0.24	**	0.01-0.36
Cr-U (µg/g creat)	1.23 ± 0.98	1.58 ± 0.83	1.12 ± 0.68	**	0.24-1.80
Cr-FN (µg/g)	1.27 ± 1.42	1.66 ± 2.82	0.95 ± 0.62		0.22-3.20
Cr-TN (µg/g)	1.19 ± 0.99	2.17 ± 2.41	0.91 ± 0.92	**	0.22-3.20
Cr-H (µg/g)	0.07 ± 0.06	0.07 ± 0.08	0.18 ± 0.33	*	0.05-0.53
Cu-B (mg/L)	1.39 ± 0.59	1.02 ± 0.24	0.95 ± 0.11	**	780-1760
Cu-H (µg/g)	19.93 ± 21.28	16.96 ± 10.60	14.73 ± 6.47		8.5-96
Fe-B (mg/L)	500.54 ± 90.83	449.10 ± 11.86	497.67 ± 47.02		390-550
Fe-H (µg/g)	12.22 ± 12.57	11.15 ± 7.65	13.94 ± 9.20		4.9-23
Hg-U (µg/g creat)	1.09 ± 0.70	1.13 ± 0.93	1.09 ± 0.89		0.14-2.21
Hg-FN (µg/g)	0.62 ± 0.34	0.61 ± 0.42	0.72 ± 0.37		0.03-0.31
Hg-TN (µg/g)	0.48 ± 0.26	0.46 ± 0.48	0.51 ± 0.36		0.03-0.31
Hg-H (µg/g)	1.58 ± 0.90	1.50 ± 1.27	1.77 ± 0.89		0.05-0.93
K-B (g/L)	2.16 ± 0.28	2.10 ± 0.11	2.08 ± 0.16		1.4-2.0
Mg-B (mg/L)	27.62 ± 4.74	30.18 ± 4.16	28.27 ± 3.44	*	31.2-34.2
Mg-FN (µg/g)	120.83 ± 40.50	161.67 ± 141.34	123.17 ± 26.59		55-191
Mg-TN (µg/g)	164.54 ± 97.02	175.55 ± 120.18	198.46 ± 129.93		55-191
Mg-H (µg/g)	51.52 ± 65.34	56.47 ± 47.84	34.17 ± 25.71		8.5-141
Mn-B (µg/L)	21.12 ± 23.14	22.35 ± 7.53	25.39 ± 31.75		5.0-12.8
Mn-U (µg/g creat)	1.51 ± 2.32	3.07 ± 2.52	1.45 ± 1.91	**	0.11-1.32
Mn-FN (µg/g)	1.63 ± 2.88	2.09 ± 2.28	1.88 ± 1.58		0.19-3.30
Mn-TN (µg/g)	1.25 ± 1.29	2.84 ± 3.17	1.98 ± 3.19	*	0.19-3.30
Mn-H (µg/g)	0.70 ± 0.84	0.77 ± 0.69	1.50 ± 1.65	*	0.08-2.41
Mo-B (µg/L)	3.75 ± 2.64	9.25 ± 8.48	6.78 ± 7.20	*	0.77-7.86
Na-B (g/L)	1.61 ± 0.22	1.59 ± 0.21	1.54 ± 0.14		3.1-3.4
Ni-U (µg/g creat)	6.14 ± 3.33	7.94 ± 6.20	7.53 ± 6.89		0.59-4.06
Ni-FN (µg/g)	2.43 ± 2.36	3.48 ± 6.10	2.10 ± 2.28		0.14-6.95
Ni-TN (µg/g)	1.24 ± 1.03	4.12 ± 9.20	0.98 ± 0.90		0.14-6.95
Ni-H (µg/g)	0.37 ± 0.25	0.30 ± 0.24	0.47 ± 0.85		0.11-1.60
Pb-B (µg/L)	36.01 ± 25.81	34.08 ± 39.39	63.72 ± 58.56	**	11.4-62.8
Pb-U (µg/g creat)	2.43 ± 2.26	2.81 ± 5.3	4.59 ± 6.82	*	0.01-2.14
Pb-FN (µg/g)	1.33 ± 0.97	1.61 ± 1.65	1.72 ± 1.28		0.27-4.75
Pb-TN (µg/g)	0.99 ± 1.08	1.25 ± 1.33	1.16 ± 1.38		0.27-4.75

Pb-H (µg/g)	1.55 ± 2.92	1.45 ± 2	3.02 ± 3.00	**	0.22-7.26
S-FN (mg/g)	30.0 ± 8.36	37.40 ± 21.79	31.87 ± 4.49		23.4-43
S-TN (g/g)	25.74 ± 7.82	28.49 ± 19.18	25.16 ± 5.93		23.4-43
S-H (mg/g)	63.85 ± 16.47	52.11 ± 14.64	57.24 ± 17.75	**	40.7-55
Se-B (µg/L)	198.44 ± 41.98	200.38 ± 39.3	226.21 ± 52.16	*	89-154
Se-U (µg/g creat)	29.17 ± 12	31.89 ± 11.82	33.14 ± 25.65		10.5-45.5
Se-FN (µg/g)	0.75 ± 0.37	1.13 ± 1.16	0.64 ± 0.28	**	0.62-1.53
Se-TN (µg/g)	0.58 ± 0.46	0.63 ± 0.35	0.51 ± 0.19		0.62-1.53
Se-H (µg/g)	0.50 ± 0.34	0.43 ± 0.16	0.49 ± 0.27		0.48-1.84
Si-B (mg/L)	1.36 ± 0.46	1.26 ± 0.40	1.29 ± 0.15		na
Zn-B (mg/L)	12.81 ± 7.44	19.74 ± 15.52	22.06 ± 17.45		3.1-9.8
Zn-FN (µg/g)	198.30 ± 111.98	199.63 ± 107.79	176.94 ± 50.05		80-191
Zn-TN (µg/g)	142.60 ± 106.74	136.49 ± 80.72	126.14 ± 43.21		80-191
Zn-H (µg/g)	158.49 ± 80.40	176.47 ± 89.28	196.30 ± 88.48	*	68-198

[a]Values in blood were obtained from Alimonti et al. (2005) and Goullé et al. (2005); values in urine were obtained from Goullé et al. (2005); values in finger nails, toe nails and hair were obtained from Rodushkin et al. (2000);

[b]Higher values can be found in smokers;

B – Blood; U – Urine; FN – Fingernails; TN – Toenails; H - Hair

na - not available;

*$P<0.05$ and **$P<0.01$ (ANOVA test)

Some of the elements – As, Cd, Cr, Hg, Mg, Mn, Mo, Ni, and Pb – presented higher values in exposed groups (environmental and/or occupational) in all the matrices when compared to controls. Among all the elements analyzed, As, Cr, Mn, and Pb showed significantly different values in three different matrices; Se in two; and Cd, Cu, Mg, Mo, S and Zn in one. No significant differences were observed for Ca, Fe, Hg, K, Na, Ni and Si in any matrix, while Cu and S presented significantly lower values in both exposed groups in blood and hair, respectively.

1.1 Comparison with reference/published ranges

When comparing these results with reference/published ranges of exposure (**Table 7**), the level of exposure in our study groups was generally higher, for some elements in some of the matrices, particularly in blood and urine samples. Concerning nails and hair samples, the vast majority of concentrations were within or relatively close to the reference ranges (except for Hg).

1.2. Effect of exposure after adjustment for confounders

Results of the multivariable analysis for the effect of exposure adjusted by age, smoking and metal-specific actual confounders, and stratified by gender in those cases for which a

significant effect modification (excluding the occupationally exposed group) was found are presented in **Table 8**.

TABLE 8. Effect of exposure on concentrations of metals. Adjustment for age, smoking and metal-specific actual confounders.

		Controls		Environmentally Exposed		Occupationally Exposed	
		Mean Ratio	[95%CI]	Mean Ratio	[95%CI]	Mean Ratio	[95%CI]
As-B (uµg/L)	O	1.00		1.38*	[1.03;1.86]	1.15	[0.84;1.58]
As-UI (µg/g creat)	M	1.00		1.13	[0.62;2.06]	1.38	[0.84;2.26]
	F	2.02*	[1.13;3.62]	2.56**	[1.45;4.49]		
As-FN (µg/g)	O	1.00		5.65**	[1.8;17.76]	9.20**	[3.09;27.36]
As-TN (µg/g)	O	1.00		3.42**	[1.37;8.52]	4.39**	[1.78;10.80]
As-H (µg/g)	O	1.00		1.36	[0.38;4.86]	2.66	[0.85;8.33]
Ca-B (µg/L)	O	1.00		0.92	[0.83;1.02]	0.96	[0.86;1.08]
Cd-B (µg/L)	O	1.00		1.02	[0.65;1.58]	1.08	[0.67;1.74]
Cd-U (µg/g creat)	O	1.00		1.29	[0.66;2.49]	0.82	[0.42;1.60]
Cd-FN (µg/g)	O	1.00		1.54	[0.40;5.95]	0.67	[0.16;2.78]
Cd-TN (µg/g)	O	1.00		1.19	[0.14;10.16]	1.46	[0.18;11.91]
Cd-H (µg/g)	O	1.00		1.81	[0.21;15.62]	4.20	[0.60;29.27]
Cr-U (µg/g creat)	O	1.00		1.16	[0.77;1.74]	0.79	[0.51;1.23]
Cr-FN (µg/g)	M	1.00		1.52	[0.65;3.52]	1.21	[0.58;2.52]
	F	2.28*	[1.05;4.93]	3.17**	[1.45;6.89]		
Cr-TN (µg/g)	M	1.00		1.34	[0.62;2.92]	1.09	[0.55;2.19]
	F	1.64	[0.77;3.47]	2.85**. §	[1.39;5.85]		
Cr-H (µg/g)	O	1.00		0.88	[0.15;4.98]	2.44	[0.53;11.25]
Cu-B (µg/L)	O	1.00		0.76**	[0.65;0.89]	0.71**	[0.60;0.85]
Cu-H (µg/g)	O	1.00		0.91	[0.68;1.22]	0.71*	[0.53;0.95]
Fe-B (µg/L)	O	1.00		0.75	[0.46;1.23]	1.13	[0.64;1.99]
Fe-H (µg/g)	M	1.00		0.98	[0.65;1.49]	0.87	[0.62;1.23]
	F	0.61*	[0.41;0.90]	0.62*	[0.41;0.93]		
Hg-U (µg/g creat)	O	1.00		0.99	[0.64;1.53]	1.07	[0.68;1.69]
Hg-FN (µg/g)	O	1.00		0.94	[0.52;1.71]	1.28	[0.70;2.35]
Hg-TN (µg/g)	O	1.00		0.81	[0.41;1.61]	1.06	[0.53;2.14]
Hg-H (µg/g)	M	1.00		1.17	[0.68;2.01]	1.08	[0.67;1.74]
	F	0.88	[0.52;1.50]	0.62	[0.35;1.09]		
K-B (µg/L)	O	1.00		0.98	[0.92;1.04]	0.98	[0.91;1.05]
Mg-B (µg/L)[INT]	M	1.00		0.97	[0.87;1.07]	0.93	[0.86;1.02]
	F	0.86**	[0.78;0.95]	1.02[§§]	[0.92;1.12]		
Mg-FN (µg/g)	O	1.00		1.36**	[1.10;1.67]	1.03	[0.84;1.26]
Mg-TN (µg/g)[INT]	M	1.00		0.78	[0.56;1.10]	0.99	[0.74;1.32]
	F	0.83	[0.59;1.16]	1.09	[0.78;1.52]		
Mg-H (µg/g)	M	1.00		0.90	[0.53;1.55]	1.15	[0.75;1.78]
	F	1.87*	[1.12;3.09]	2.34**	[1.40;3.90]		

Mn-B (µg/L)	O	1.00		1.10	[0.80;1.51]	1.06	[0.74;1.50]
Mn-U (µg/g creat)	O	1.00		1.86**	[1.20;2.90]	0.92	[0.56;1.52]
Mn-FN (µg/g)	O	1.00		1.31	[0.82;2.10]	1.10	[0.69;1.75]
Mn-TN (µg/g)	M	1.00		1.25	[0.56;2.78]	1.41	[0.73;2.70]
	F	1.49	[0.67;3.28]	4.07**.§§	[1.93;8.59]		
Mn-H (µg/g)	O	1.00		1.12	[0.65;1.92]	1.88**	[1.17;3.03]
Mo-B (µg/L)	O	1.00		2.83**	[1.85;4.32]	1.42	[0.90;2.23]
Na-B (µg/L)	O	1.00		0.92	[0.84;1.01]	0.95	[0.85;1.05]
Ni-U (µg/g creat)	O	1.00		1.29	[0.95;1.75]	1.17	[0.85;1.60]
Ni-FN (µg/g)	O	1.00		1.49	[0.91;2.44]	1.38	[0.78;2.43]
Ni-TN (µg/g)[INT]	M	1.00		1.08	[0.42;2.73]	1.20	[0.53;2.72]
	F	1.06	[0.41;2.70]	4.76**.§§	[2.05;11.05]		
Ni-H (µg/g)	O	1.00		0.81	[0.37;1.76]	1.47	[0.68;3.16]
Pb-B (µg/L)	M	1.00		1.09	[0.70;1.71]	1.19	[0.83;1.71]
	F	0.65*	[0.43;0.98]	0.68	[0.44;1.05]		
Pb-U (µg/g creat)	O	1.00		1.16	[0.71;1.90]	1.46	[0.91;2.33]
Pb-FN (µg/g)	O	1.00		1.42	[0.95;2.11]	1.28	[0.86;1.89]
Pb-TN (µg/g)	O	1.00		1.13	[0.72;1.77]	1.07	[0.67;1.73]
Pb-H (µg/g)	O	1.00		1.27	[0.73;2.21]	2.17**	[1.27;3.71]
S-FN (µg/g)	O	1.00		1.26*	[1.05;1.50]	1.13	[0.94;1.35]
S-TN (µg/g)	O	1.00		1.01	[0.83;1.22]	1.03	[0.84;1.26]
S-H (µg/g)	M	1.00		0.83	[0.65;1.06]	0.85	[0.70;1.05]
	F	0.86	[0.68;1.08]	0.72**	[0.57;0.90]		
Se-B (µg/L)	O	1.00		1.01	[0.88;1.56]	1.17*	[1.02;1.34]
Se-U (µg/g creat)	M	1.00		0.96	[0.69;1.33]	1.17	[0.89;1.52]
	F	1.15	[0.85;1.57]	1.30	[0.95;1.77]		
Se-FN (µg/g)	O	1.00		1.54	[0.93;2.55]	0.90	[0.50;1.60]
Se-TN (µg/g)	O	1.00		1.10	[0.60;2.00]	0.89	[0.46;1.73]
Se-H (µg/g)	O	1.00		0.91	[0.45;1.84]	0.91	[0.46;1.78]
Si-B (µg/L)	O	1.00		0.91	[0.73;1.13]	0.96	[0.74;1.23]
Zn-B (µg/L)	M	1.00		2.15**	[1.37;3.40]	1.53*	[1.05;2.23]
	F	0.92	[0.60;1.41]	1.13	[0.74;1.73]		
Zn-FN (µg/g)	O	1.00		1.07	[0.89;1.27]	1.00	[0.82;1.21]
Zn-TN (µg/g)	O	1.00		0.96	[0.78;1.17]	0.90	[0.73;1.12]
Zn-H (µg/g)	O	1.00		1.23*	[1.02;1.48]	1.34**	[1.11;1.61]

Comparison versus reference level (control group or males control group): *$P<0.05$; **$P<0.01$;

Comparison versus females control group: § P<0.05; §§ P<0.01;

Comparison versus males environmentally exposed group: †P<0.05; ††P<0.01;

O – overall (overall effect indicates no effect of gender); F – females; M – males;

INT - indicates gender-by-exposure interaction;

B – Blood; U – Urine; FN – Fingernails; TN – Toenails; H – Hair.

After taking into account all possible confounding factors, elements which still presented significantly higher values in the exposed groups in one or more biological matrices were As, Cr, Mg, Mn, Mo, Ni, Pb, S, Se and Zn. Copper was the only element showing

significantly lower values in the exposed groups, both in blood and hair. All significant differences were obtained in the groups with environmental or both, environmental and occupational exposure, except for Cu, Mn and Pb in hair, and Se in blood, which significantly differed from controls only in the occupationally exposed group. No significant differences were obtained for Cd, Fe, Hg, and Si. For some of these elements the significant differences were only obtained for one of the genders, namely Cr in toenails (only in females), Mg in blood (only in females), Mn in toenails (only in females), Ni in toenails (only in females), and Zn in blood (only in males).

1.3 Correlations between matrices

Correlations between the concentration of the same metal(loid) in different matrices were analysed and the results are presented in **Table 9** (only significant results are shown).

TABLE 9. Correlation coefficients between the same metal in different matrices (only statistically significant results).

	B - U	B - FN	B - TN	U - FN	U - TN	U - H	FN - TN	FN - H	TN - H
As	0.375**	0.314**	0.282*	0.298**	0.220*	0.183*	0.487**	0.246**	0.216*
Cd	0.294*								
Hg				0.219*		0.311**	0.519**	0.489**	0.441**
Mg	0.224*						0.224*		0.234*
Mn							0.227*	0.188*	
Ni							0.320**		
Pb	0.487**						0.241*	0.306**	
S							0.201*		
Se							0.327**		

Grey cells: one of the matrices was not analyzed.
*$P<0.05$; **$P<0.01$

Only As, Hg, Mg, Mn and Pb presented more than one significant and positive correlation between two or more matrices, and among these elements only As and Hg showed correlations between blood or urine and fingernails, toenails or hair. Fingernail and toenail samples were significantly correlated in all elements shown but Cd. Significant positive correlations were also obtained between fingernails and hair for As, Hg, Mn and Pb, and between toenails and hair for As, Hg and Mg.

1.4 Effect of gender, age and smoking habits

The effects of gender, age and smoking habits on the levels of metal(loid)s in the different matrices were also evaluated and the results are presented in **Table 10**.

TABLE 10. Effect of gender, age and smoking on the levels of metals in the different biological samples (only significant effect on at least one parameter are shown).

	Gender (effect in males)	Age (effect with regard to 25-50 years)			Smoking (effect in smokers)
		51-60 years	61-70 years	≥71 years	
As-U (µg/g creat)	↓		↑	↑↑	
As-TN (µg/g)				↓	
Cr-U (µg/g creat)			↑		
Cr-FN (µg/g)					
Cr-TN (µg/g)	↓				
Cu-B (µg/L)		↓	↓		
Cu-H (µg/g)					↑↑
Fe-H (µg/g)	↑↑	↑		↑↑	
Hg-H (µg/g)	↑				↓
K-B (µg/L)		↑			
Mg-B (µg/L)	↑			↑	
Mg-TN (µg/g)				↓↓	↑
Mg-H (µg/g)	↓↓			↓	
Mn-TN (µg/g)	↓	↑	↑↑	↑↑	↑
Pb-B (µg/L)	↑↑	↑			↑
S-H (µg/g)	↑				
Se-U (µg/g creat)	↓↓			↑	
Zn-B (µg/L)	↑↑				
Zn-FN (µg/g)		↓↓	↓		
Zn-TN (µg/g)				↓	
Zn-H (µg/g)		↓↓		↓	

Arrows up indicate increase, arrows down indicate decrease;

One arrow: $P<0.05$; Two arrows: $P<0.01$;

Concerning gender, the majority of the elements showed significantly higher values in males in blood and hair samples (except for Mg in hair, higher in females), and significantly higher values in females in urine, fingernails and toenail.

Regarding age, the effects depended mainly on the matrix. All elements showed a significant increase in older groups in blood and urine samples, except Cu in blood, and a significant decrease with age in fingernails, toenails and hair samples, except for Mn in toenails and Fe in hair.

As regards smoking, significantly higher levels were generally observed in smokers in the different matrices, except for Hg in hair which was significantly lower. These results are also shown schematically in **Figure 7**.
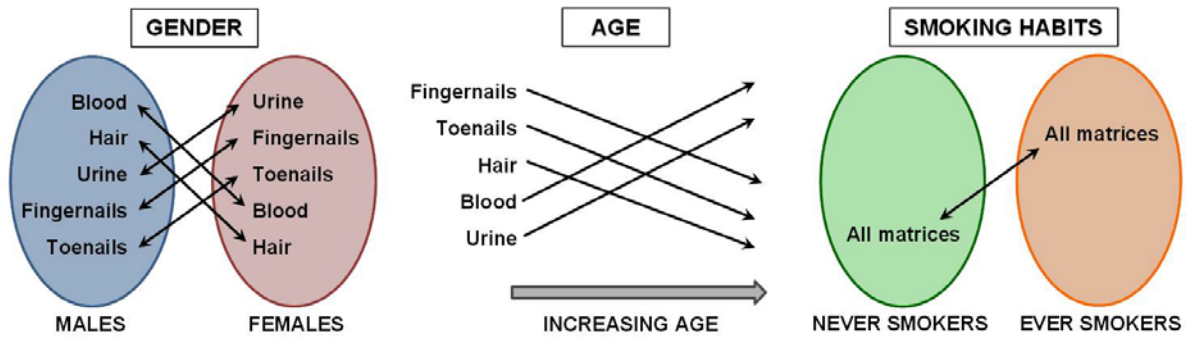
FIGURE 7. Schematic representation of the effect of gender, age and smoking on the concentration of metal(loid)s in the different matrices. Variations in the relative concentration of metal(loid)s are represented horizontally.

2. BIOMARKERS OF EFFECT

2.1 Biomarkers of Genotoxicity

Univariate comparisons of genotoxicity biomarkers by study group are reported in **Table 11**.

TABLE 11. Levels of genotoxicity biomarkers in the study groups.

	Controls		Environmentally Exposed		Occupationally Exposed		
	N	mean ± SD	*N*	mean ± SD	*N*	mean ± SD	*P-value**
MN (‰)	40	6.45 ± 4.47	41	8.46 ± 5.27	41	4.98 ± 3.06	**0.002**
TCR-Mf (10^{-4})	39	3.80 ± 2.11	34	4.92 ± 3.86	38	5.80 ± 3.93	**0.018**
%DNAT	40	12.40 ± 3.04	41	24.58 ± 7.75	41	18.73 ± 7.60	**< 0.001**
CA-total	40	2.65 ± 2.11	41	5.56 ± 2.92	41	3.24 ± 2.45	**< 0.001**
CA-cromosome_type	40	0.55 ± 1.04	41	1.22 ± 1.39	41	0.71 ± 1.10	**0.018**
CA-chromatide_type	40	2.10 ± 1.52	41	4.34 ± 2.56	41	2.54 ± 1.90	**< 0.001**
Aneuploidies (45)	40	1.65 ± 1.46	41	3.32 ± 1.77	41	2.66 ± 1.51	**< 0.001**
Aneuploidies (47)	40	0.13 ± 0.33	41	0.20 ± 0.46	41	0.32 ± 0.57	0.221
Gaps-single	40	0.83 ± 1.03	41	2.15 ± 1.86	41	1.39 ± 1.51	**0.001**
Gaps-double	40	0.00 ± 0.00	41	0.37 ± 0.54	41	0.17 ± 0.44	**< 0.001**

*ANOVA test

Significant differences were found for all biomarkers but for the frequency of aneuploidies, with higher values in the exposed groups, particularly in the environmentally exposed.

2.1.1 Effect of exposure, age and smoking habits

The genotoxic effect of exposure, age, and smoking habits, adjusted for the presence of confounding, was evaluated with multivariate modeling (**Table 12**).

TABLE 12. Effect of exposure, age and smoking habits on the biomarkers of genotoxicity. Adjustment for age, smoking and genotoxicity parameter-specific actual confounders.

	TCR-Mf		%DNAT		MN		CA-total		CA-chromosome		CA-chromatide	
	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]
Exposure												
Controls (N=40)	1.00		1.00		1.00		1.00		1.00		1.00	
Env. Exposed (N=41)	0.99	[0.75;1.32]	1.78**	[1.54;2.06]	1.21	[0.89;1.64]	2.15**	[1.57;2.93]	2.68**	[1.52;4.72]	2.05**	[1.53;2.73]
Occup.Exposed (N=41)	1.38*	[1.04;1.82]	1.45**	[1.25;1.68]	0.75	[0.54;1.02]	1.12	[0.81;1.56]	1.23	[0.67;2.26]	1.24	[0.91;1.69]
Age (years)												
25-50 (N=23)	1.00		1.00		1.00		1.00		1.00		1.00	
51-60 (N=35)	1.30	[0.93;1.82]	0.96	[0.80;1.14]	1.35	[0.92;1.99]	1.20	[0.82;1.77]	1.73	[0.90;3.31]	1.09	[0.75;1.58]
61-70 (N=38)	1.78**	[1.28;2.48]	1.13	[0.95;1.34]	1.17	[0.80;1.68]	1.20	[0.84;1.72]	1.04	[0.57;1.91]	1.24	[0.87;1.76]
>71 (N=26)	1.28	[0.89;1.83]	1.30**	[1.09;1.56]	1.14	[0.77;1.68]	1.27	[0.87;1.87]	0.94	[0.47;1.88]	1.30	[0.92;1.84]
Smoking habits												
Never smokers (N=73)	1.00		1.00		1.00		1.00		1.00		1.00	
Ever smokers (N=49)	0.88	[0.69;1.12]	1.09	[0.96;1.23]	0.81	[0.61;1.07]	1.13	[0.87;1.48]	1.02	[0.64;1.62]	1.21	[0.94;1.56]

MR – Mean Ratio

*$P<0.05$, **$P<0.01$

Significant increases observed in exposed groups when compared to controls confirmed the result of univariate analysis, with the only exception of MN which did not show any significant differences. The environmentally exposed group showed significantly higher %DNAT, CA-total, CA-chromosome and CA-chromatid when compared to the control group. The occupationally exposed group showed significantly higher TCR-Mf and %DNAT.

Significant effect of age was observed for TCR-Mf in the 61-70 years age-class and also for %DNAT in individuals older than 71 years (>71).

No significant effect of smoking habits was observed on any biomarker. Higher mean ratios (MR) were generally observed in individuals living in the polluted villages than in those who worked in the mine.

2.1.2 Effect of gender

The effect of gender was evaluated only in the group of exposed residents and in controls, since miners were only males. Not surprisingly, only MN frequency was influenced by this factor, with females showing significantly higher frequencies than males in controls and in the environmentally exposed group (**Table 13**).

TABLE 13. Effect of gender and exposure on MN, excluding the occupationally exposed population.

	Controls		Environmentally Exposed	
	MR	**[95% CI]**	**MR**	**[95% CI]**
Males	1.00		0.93	[0.59;1.47]
	(n=17)		(n=16)	
Females	1.78**	[1.78;2.69]	1.51	[0.89;2.56]
	(n=23)		(n=25)	

MR – Mean Ratio

*P<0.01, significant difference with regard to control males.

The effect of exposure in this biomarker was revealed by the increased MR in females environmentally exposed as compared to non exposed females (MR: 1.41; 95% CI: 1.00-1.97). No significant differences between males were observed.

2.1.3 Synergistic effect of environmental and occupational exposure

The presence of synergy between occupational and environmental exposure was evaluated comparing miners living in polluted villages vs. those living in villages upstream the mine. No biomarker showed higher frequency in the group of subjects exposed to metal(oid)s from both sources (**Table 14**).

TABLE **14.** Effect of exposure in 4 categories (taking into account the origin village of the occupationally exposed individuals) on TCR-MF, %DNAT and CA-total. Adjustment for age, smoking and genotoxicity parameter-specific actual confounders.

	TCR-Mf		%DNAT		CA-total	
	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]
Controls (N=40)	1.00		1.00		1.00	
EE (N=41)	0.99	[0.74;1.31]	1.80**	[1.57;2.07]	2.24**	[1.66;3.02]
OEO (N=24)	1.49*	[1.08;2.05]	1.21*	[1.04;1.42]	0.82	[0.56;1.20]
O+EE (N=17)	1.20	[0.82;1.77]	1.87**	[1.57;2.22]	1.62*	[1.11;2.38]

EE: environmentally exposed; OEO: occupationally exposed only; O+EE: occupationally + environmentally exposed.

MR – Mean Ratio

*P<0.05, **P<0.01

2.1.4. Effect of metal(loid) concentration

To evaluate the effect of metal(loid)s concentration on the selected biomarkers we used measure of individual exposure for our subjects (biomarkers of exposure).

Subjects were divided according to the tertile distribution of each metal(loid) level, in all biological matrices analysed (blood, urine, hair, fingernails, and toenails). Most specific results were obtained for As, Mn and Pb in toenails (TN) (**Table 15**).

TABLE 15. Effect of the levels of As, Mn, and Pb in toenails on the genotoxicity parameters. Adjustment for age, smoking and genotoxicity parameter-specific actual confounders.

	TCR-Mf		%DNAT		MN		CA-total		CA-chromosome		CA-chromatide	
	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]
As-TN												
1st tertile	1.00		1.00		1.00		1.00		1.00		1.00	
2nd tertile	0.86	[0.75;1.32]	1.03	[0.88;1.21]	1.13	[0.84;1.53]	1.16	[0.83;1.62]	2.07	[0.90;4.78]	1.06	[0.75;1.49]
3rd tertile	0.96	[1.04;1.82]	1.42**	[1.22;1.66]	1.67**	[1.28;2.20]	1.96**	[1.44;2.68]	4.57**	[2.10;9.95]	1.57**	[1.14;2.17]
Mn-TN												
1st tertile	1.00		1.00		1.00		1.00		1.00		1.00	
2nd tertile	1.08	[0.81;1.45]	1.01	[0.84;1.21]	1.23	[0.95;1.71]	1.32	[0.94;1.86]	1.71	[0.82;3.56]	1.09	[0.78;1.54]
3rd tertile	1.02	[0.76;1.38]	1.18	[0.99;1.40]	1.41**	[1.07;1.87]	1.72*	[1.22;2.41]	2.18**	[1.05;4.51]	1.31	[0.95;1.82]
Pb-TN												
1st tertile	1.00		1.00		1.00		1.00		1.00		1.00	
2nd tertile	1.05	[0.93;1.82]	1.11	[0.93;1.34]	1.25	[0.92;1.71]	1.54**	[1.10;2.16]	1.93	[0.89;4.18]	1.49*	[1.05;2.12]
3rd tertile	1.36	[0.99;1.89]	1.11	[0.92;1.33]	1.16	[0.86;1.56]	1.43*	[2.00;1.03]	2.58**	[1.26;5.30]	1.24	[0.87;1.75]

MR – Mean Ratio

*$P<0.05$, **$P<0.01$

Levels of As in the 3rd tertile were significantly associated with the level of %DNAT, MN, CA-total, CA-chromosome, and CA-chromatid. Mn levels in the 3rd tertile determined a significant increase of MN, CA-total and CA-chromosome. Higher levels of Pb were generally associated with higher frequencies of CA-total, CA-chromosome and CA-chomatid.

2.2 Immune markers

Results concerning the level of immunotoxicity biomarkers in the study groups are presented in **Table 16**.

TABLE 16. Levels of immunotoxicity biomarkers in the study groups.

	Controls		Environmentally Exposed		Occupationally Exposed		
	N	mean ± SD	N	mean ± SD	N	mean ± SD	*P-value**
Neopterin (nmol/L)	24	4.35 ± 0.83	22	4.77 ± 0.78	32	4.90 ± 1.63	0.255
Tryptophan (µmol/L)	24	51.24 ± 7.73	22	51.95 ± 7.63	32	52.76 ± 8.13	0.773
Kynurenine (µmol/L)	24	1.80 ± 0.38	22	2.04 ± 0.6	32	1.93 ± 0.49	0.277
Kyn/Trp (µmol/mmol)	24	35.42 ± 6.14	22	39.26 ± 10.04	32	37.13 ± 10.13	0.361
Nitrite (µmol)	24	23.44 ± 20.50	22	16.54 ± 12.26	32	32.87 ± 32.16	0.112
%CD3$^+$	40	75.46 ± 8.85	35	70.6 ± 9.99	38	68.88 ± 12.93	**0.022**
%CD4$^+$	40	47.18 ± 6.92	35	46.59 ± 8.82	38	40.07 ± 9.84	**0.001**
%CD8$^+$	40	25.98 ± 9.52	34	20.93 ± 6.27	38	26.62 ± 11.17	**0.022**
CD4$^+$/CD8$^+$	40	2.01 ± 0.65	34	2.40 ± 0.84	38	1.87 ± 1.11	**0.037**
%CD19$^+$	40	8.77 ± 6.91	35	7.68 ± 3.27	38	7.15 ± 3.55	0.339
%CD16$^+$56$^+$	40	13.65 ± 6.75	35	17.19 ± 7.72	38	20.19 ± 11.90	**0.008**

*ANOVA test

Significant differences were observed in the univariate analysis for %CD3$^+$, %CD4$^+$, %CD8$^+$, %CD16$^+$56$^+$, and CD4$^+$/CD8$^+$ ratio. No significant differences were obtained for %CD19$^+$ and the levels of neopterin, tryptophan, kynurenine, Kyn/Trp and nitrite among the three groups.

2.2.1 Effect of exposure, age and smoking habits

When multivariate modelling was applied, levels of neopterin, kynurenine and Kyn/Trp were found to be influenced by age (**Table 17**), with significantly higher MR in older individuals, when compared to the youngest group (25-50 years).

TABLE 17. Effect of exposure on neopterin, tryptophan, kynurenin and nitrite concentrations stratified by exposure, age, and smoking habits. All models were adjustment for parameter-specific actual confounders.

	Neopterin		Tryptophan		Kynurenine		Kyn/Trp		Nitrite	
	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]
Exposure										
Controls (N=40)	1.00		1.00		1.00		1.00		1.00	
Env. Exposed (N=41)	1.02	[0.90;1.17]	1.03	[0.93;1.14]	1.11	[0.95;1.28]	1.07	[0.94;1.23]	0.73	[0.43;1.20]
Occup. Exposed (N=41)	1.03	[0.91;1.17]	1.03	[0.94;1.13]	0.96	[0.84;1.11]	0.93	[0.82;1.07]	0.95	[0.58;1.56]
Age (years)										
25-50 (N=23)	1.00		1.00		1.00		1.00		1.00	
51-60 (N=35)	1.11	[0.95;1.30]	1.02	[0.90;1.15]	1.40**	[1.17;1.69]	1.38**	[1.16;1.63]	1.75	[0.93;3.31]
61-70 (N=38)	1.21*	[1.03;1.42]	0.98	[0.87;1.11]	1.28*	[1.06;1.55]	1.31**	[1.10;1.56]	1.62	[0.85;3.08]
>71 (N=26)	1.44**	[1.21;1.72]	0.97	[0.85;1.11]	1.59**	[1.30;1.95]	1.63**	[1.36;1.97]	1.33	[0.67;2.66]
Smoking habits										
Never smokers (N=73)	1.00		1.00		1.00		1.00		1.00	
Ever smokers (N=49)	1.11	[0.99;1.23]	0.98	[0.90;1.06]	1.00	[0.88;1.13]	1.02	[0.91;1.14]	1.19	[0.78;1.82]

MR – Mean Ratio

*P<0.05, **P<0.01

No significant effect of exposure or smoking habits was observed on any of these markers, but significant correlations were obtained for neopterin with kynurenine (r=0.569, P<0.01) and Kyn/Trp (r=0.616, P<0.01).

Significant effects of exposure and age were observed on the percentage of the different lymphocyte subsets (**Table 18**).

TABLE 18. Effect of exposure on lymphocytes subpopulations stratified by exposure, age, and smoking habits. All models were adjustment for parameter-specific actual confounders.

	%CD3$^+$		%CD4$^+$		%CD8$^+$		CD4$^+$/CD8$^+$		%CD19$^+$		%CD16$^+$56$^+$	
	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]
Exposure												
Controls (N=40)	1.00		1.00		1.00		1.00		1.00		1.00	
Env. Exposed (N=41)	0.96	[0.88;1.04]	1.00	[0.90;1.11]	0.80*	[0.67;0.96]	1.27*	[1.02;1.58]	0.78	[0.59;1.02]	1.29	[0.99;1.68]
Occup. Exposed (N=41)	0.92*	[0.85;0.99]	0.84**	[0.76;0.93]	0.96	[0.79;1.15]	0.88	[0.70;1.11]	0.92	[0.69;1.23]	1.44**	[1.10;1.88]
Age (years)												
25-50 (N=23)	1.00		1.00		1.00		1.00		1.00		1.00	
51-60 (N=35)	0.97	[0.88;1.07]	0.96	[0.85;1.09]	0.98	[0.79;1.21]	1.01	[0.78;1.30]	0.79	[0.58;1.09]	1.09	[0.79;1.50]
61-70 (N=38)	0.90*	[0.82;0.99]	0.95	[0.85;1.07]	0.79*	[0.64;0.98]	1.23	[0.96;1.59]	1.07	[0.78;1.46]	1.33	[0.98;1.81]
>71 (N=26)	0.92	[0.83;1.03]	0.90	[0.79;1.02]	0.98	[0.71;1.12]	1.02	[0.77;1.33]	0.71*	[0.51;0.99]	1.31	[0.94;1.82]
Smoking habits												
Never smokers (N=73)	1.00		1.00		1.00		1.00		1.00		1.00	
Ever smokers (N=49)	0.99	[0.93;1.07]	1.04	[0.95;1.13]	1.02	[0.87;1.20]	0.98	[0.81;1.19]	0.87	[0.70;1.09]	0.93	[0.74;1.16]

MR – Mean Ratio

*P<0.05, **P<0.01

In the environmentally exposed group we observed MR significantly lower for %CD8$^+$ and higher for CD4$^+$/CD8$^+$ ratio when compared to controls. As for the occupationally exposed population, significant decrease was obtained in %CD3$^+$ and %CD4$^+$, and significant increase in %CD16$^+$56$^+$. Additionally, MR of %CD3$^+$, %CD8$^+$ and %CD19$^+$ were significantly lower in older individuals, when compared to the youngest group (25-50 years). No significant effect of smoking habits was observed on any of these markers.

2.2.2 Effect of gender

The effect of gender was also investigated excluding the occupationally exposed group, composed only by males. The only parameter significantly influenced by this factor, and only in exposed group, was %CD19$^+$, which showed a significantly lower MR in males from the environmentally exposed group when compared to control females (**Table 19**).

TABLE 19. Effect of gender and exposure on %CD19$^+$, excluding the occupationally exposed population.

	Controls		Environmentally Exposed	
	MR	**[95% CI]**	**MR**	**[95% CI]**
Females	1.00		0.99	[0.72;1.38]
	(*N*=23)		(*N*=23)	
Males	0.85	[0.58;1.27]	0.60*	[0.37;0.98]
	(*N*=17)		(*N*=12)	

MR – Mean Ratio
*P<0.01, significant difference with regard to control females.

2.2.3 Effect of other confounders

Since populations living in the small villages around the mining site are strongly dependent on agriculture and farming, we included variables such as agricultural practices, pesticides usage, source of water for consumption and agriculture, and frequency of fish and shellfish consumption [as possible sources of metal(loid)s contamination] on the multivariate analyses. The only notable but non significant

(*P*=0.062) influence observed was that drinking bottled water was related to lower neopterin MR when compared to drinking tap water.

2.2.4 Effect of metal(loid) concentration

To quantitatively evaluate the immunotoxic effect of metal(loid)s exposure on the selected biomarkers, we associated these latter data with metal(loid)s concentrations in the biological matrices (blood, urine, finger and toe nails, and hair).

Subjects were divided according to the tertile distribution of each metal(loid) level in each biological matrix. Higher MR for neopterin, kynurenine and Kyn/Trp were observed with increasing concentrations of Pb in toenails; significance was obtained for kynurenine in the 2nd and 3rd tertiles and for K/T in the 3rd tertile, whereas the MR of neopterin in the 3rd tertile was borderline significant (*P*=0.067) (**Table 20**).

Table 20. Effect of the levels of Pb in toe nails on Neo, Kyn and K/T levels . Adjustment for age, smoking and parameter-specific actual confounders.

	Neopterin		Kynurenin		Kyn/Trp	
	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]
1st tertile	1.00		1.00		1.00	
2nd tertile	1.10	[0.97;1.24]	1.25*	[1.07;1.47]	1.08	[0.95;1.23]
3rd tertile	1.12	[0.99;1.26]	1.34*	[1.16;1.56]	1.28*	[1.14;1.44]

MR – Mean Ratio
*P<0.01

Levels of Mn in the 2nd tertile were significantly associated with decrease in %CD8$^+$ and increase in CD4$^+$/CD8$^+$, and in the 3rd tertile with increase in CD4$^+$/CD8$^+$ and decrease in %CD19$^+$ (**Table 21**).

Table 21. Effect of the levels of Mn in blood on %CD8$^+$, CD4$^+$/CD8$^+$ and %CD19$^+$ lymphocytes. Adjustment for age, smoking and immunotoxicity parameter-specific actual confounders

	%CD8$^+$		CD4$^+$/CD8$^+$		%CD19$^+$	
	MR	[95% CI]	MR	[95% CI]	MR	[95% CI]
1st tertile	1.00		1.00		1.00	
2nd tertile	0.82*	[0.68;0.97]	1.33*	[1.07;1.65]	0.98	[0.70;1.37]
3rd tertile	0.87	[0.72;1.05]	1.28*	[1.03;1.59]	0.71*	[0.51;0.99]

MR – Mean Ratio
*P<0.05

2.3 Symptomatology

We also investigated the presence of differences among groups concerning the report of symptoms related to metal(loid)s intoxication (**Table 22**). Significant differences were obtained for cough with expectoration and taking medicines, with lower frequency rates in both exposed groups. Moreover, as expected, significant influence of smoking was observed in cough with expectoration (higher frequency rate in ever smokers) and of age in sick in recent years and taking medicines (higher frequency rates in older individuals) (data not shown).

TABLE 22. Linear regression analysis for symptoms. Adjustment for age, smoking and symptom-specific actual confounders.

	Visits to doctor - sickness		Cough with expectoration		Alterations in skin		Alterations in smell	
	MR	**[95% CI]**	**MR**	**[95% CI]**	**MR**	**[95% CI]**	**MR**	**[95% CI]**
Controls (N=40)	1.00		1.00		1.00		1.00	
Env. Exposed (N=41)	1.27	[0.47;3.41]	0.27*	[0.09;0.82]	0.69	[0.15;3.06]	0.37	[0.05;2.50]
Occup. Exposed (N=41)	0.38	[0.14;1.06]	0.31*	[0.10;0.93]	0.33	[0.05;2.05]	0.68	[0.15;3.08]

	Alterations in taste		Red eyes when windy		Sick in recent years		Taking medicines	
	MR	**[95% CI]**	**MR**	**[95% CI]**	**MR**	**[95% CI]**	**MR**	**[95% CI]**
Controls (N=40)	1.00		1.00		1.00		1.00	
Env. Exposed (N=41)	0.48	[0.05;5.21]	1.75	[0.64;4.83]	0.97	[0.36;2.64]	0.52	[0.13;2.00]
Occup. Exposed (N=41)	0.37	[0.04;3.68]	0.50	[0.19;1.34]	0.62	[0.23;1.72]	0.22*	[0.06;0.85]

MR – Mean Ratio

*$P<0.05$

3. BIOMARKERS OF SUSCEPTIBILITY

The results of genotyping of polymorphisms in genes involved in the metabolism and DNA repair in study populations are presented in **Table 23**.

Table 23. Frequency of genotypes in study populations.

	All		Controls		Env. Exposed		Occup. Exposed		P-Value
	N	%	*N*	%	*N*	%	*N*	%	*
***GSTA2* rs2180314**									
G/G	26	21.3	9	22.5	8	19.5	9	22.0	
G/C	69	56.6	24	60.0	27	65.9	18	43.9	0.405
C/C	9	22.1	7	17.5	6	14.6	14	34.1	
***GSTM1* deletion**									
Positive	44	36.1	16	40.0	14	34.1	14	34.1	0.823
Null	78	63.9	24	60.0	27	65.9	27	65.9	
***GSTT1* deletion**									
Positive	88	72.1	31	77.5	30	73.2	27	65.9	0.503
Null	34	27.9	9	22.5	11	26.8	14	34.1	
***GSTP1* rs1695**									
A/A	69	56.6	19	47.5	24	58.5	26	63.4	
A/G	44	36.1	19	47.5	14	34.1	11	26.8	0.701
G/G	9	7.4	2	5.0	3	7.3	4	9.8	
***XRCC1* rs1799782**									
C/C	111	91.0	37	92.5	38	92.7	36	87.8	
C/T	2	1.6	1	2.5	-	-	1	2.4	0.709
T/T	9	7.4	2	5.0	3	7.3	4	9.8	
***XRCC1* rs25487**									
G/G	46	37.7	15	37.5	14	34.1	17	41.5	
G/A	53	43.4	20	50.0	17	41.5	16	39.0	0.613
A/A	23	18.9	5	12.5	10	24.4	8	19.5	
***APEX1* rs1130409**									
T/T	35	28.7	11	27.5	12	29.3	12	29.3	
T/G	44	36.1	14	35.0	15	36.6	15	36.6	0.947
G/G	43	35.2	15	37.5	14	34.1	14	34.1	
***MPG* rs3176383**									
A/A	122	100.0	40	100.0	41	100.0	41	100.0	
A/C	-	-	-	-	-	-	-	-	-
C/C	-	-	-	-	-	-	-	-	
***MUTYH* rs3219489**									
C/C	73	59.8	22	55.0	26	63.4	25	61.0	
C/G	43	35.2	17	42.5	13	31.7	13	31.7	0.889
G/G	6	4.9	1	2.5	2	4.9	3	7.3	

OGG1 rs1052133									
	C/C	75	61.5	23	57.5	28	63.3	24	58.5	
	C/G	43	35.2	14	35.0	13	31.7	16	39.0	0.325
	G/G	4	3.3	3	7.5	-	-	1	2.4	
PARP1 rs1136410									
	T/T	84	68.9	32	80.0	27	65.9	25	61.0	
	T/C	38	31.1	8	20.0	14	34.1	16	39.0	0.162
	C/C	-	-	-	-	-	-	-	-	
PARP4 rs13428									
	C/C	62	50.8	18	45.0	23	56.1	21	51.2	
	C/G	43	35.2	14	35.0	15	36.6	14	34.1	0.332
	G/G	17	13.9	8	20.0	3	14.6	6	14.6	
PARP4 rs1050112									
	C/C	61	50.0	18	45.0	23	56.1	20	48.8	
	C/A	45	36.9	15	37.5	15	36.6	15	36.6	0.384
	A/A	16	13.1	7	17.5	3	7.3	6	14.6	
ERCC1 rs3212986									
	G/G	60	49.2	23	57.5	21	51.5	16	39.0	
	G/T	49	40.2	16	40.0	16	39.0	17	41.5	0.056
	T/T	13	10.7	1	2.5	4	9.8	8	19.5	
ERCC4 rs1800067									
	G/G	89	73.0	29	72.5	30	73.2	30	73.2	
	G/A	30	24.6	10	25.0	11	26.8	9	22.0	0.909
	A/A	3	2.5	1	2.5	-	-	2	4.9	
ERCC5 rs17655									
	G/G	41	33.6	20	50.0	9	22.0	12	29.3	
	G/C	62	50.8	18	45.0	26	63.4	18	43.9	**0.008**
	C/C	19	15.6	2	5.0	6	14.6	11	26.8	
ERCC5 rs2227869									
	G/G	112	91.8	39	97.5	36	87.8	37	90.2	
	G/C	10	8.2	1	2.5	5	12.2	4	9.8	0.260
	C/C	-	-	-	-	-	-	-	-	

*ANOVA test

The frequencies of studied genotypes in all subjects are consistent with the findings of other authors for Caucasian (Garte and Gaspari, 2001; Laffon et al., 2006) and more specifically for Portuguese populations (Costa et al., 2008; Garcia-Lestón et. al., 2012; Gaspar et al., 2004; Silva et. al, 2009; Teixeira et al., 2004).

There was no significant difference between study groups in the frequency of studied polymorphisms except for *ERCC5* rs17655.

The influence of the polymorphisms of genes encoding for metabolic and DNA repair enzymes on the level of genotoxicity markers was evaluated, and statistically significant results are gathered in **Table 24**.

Table 24. Influence of biomarkers of susceptibility on genotoxicity parameters (only models showing significant effect are included). Adjustment for age, smoking and genotoxicity parameter-specific actual confounders.

	Control			Environmentally Exposed			Occupationally Exposed		
	N	Mean Ratio	[95%CI]	N	Mean Ratio	[95%CI]	N	Mean Ratio	[95%CI]
GSTM1 deletion									
CA-total									
Positive	16	1		14	2.31*	[1.44,3.70]	14	1.78*	[1.11,2.86]
Null	24	1.11	[0.71,1.75]	27	0.90	[0.50,1.60]	27	0.46*	[0.25,0.85]
CA-chromosome									
Positive	16	1		14	7.17**	[2.38,21.62]	14	3.88*	[1.26,11.6
Null	24	3.02*	[1.01,9.04]	27	0.25*	[0.07,0.87]	27	0.16*	[0.04,0.61]
APEX1 rs1130409									
%DNAT									
TT	11	1		12	1.91**	[1.48,2.46]	12	1.22	[0.94,1.57]
TG	14	1.02	[0.80,1.29]	15	0.83	[0.60,1.16]	15	1.40*	[1.01,1.95]
GG	15	0.90	[0.71,1.13]	14	1.01	[0.72,1.41]	14	1.13	[0.81,1.58]
OGG1 rs1052133									
CA-Total									
CC	23	1		28	2.58**	[1.74,3.84]	24	1.79**	[1.17,2.74]
CG+GG	17	1.81*	[1.15,2.86]	13	0.70	[0.40,1.24]	17	0.34**	[0.18,0.65]
CA-chromatid									
CC	23	1		28	2.38**	[1.64,3.47]	24	1.84**	[1.22,2.76]
CG+GG	17	1.65*	[1.04,2.61]	13	0.75	[0.43,1.31]	17	0.33**	[0.17,0.61]
ERCC1 rs3212986									
%DNA									
GG	23	1		21	1.48**	[1.24,1.77]	16	1.19	[0.99,1.43]
GT+TT	17	0.93	[0.78,1.13]	20	1.47**	[1.13,1.89]	25	1.44**	[1.11,1.87]
CA-chromosome									
GG	23	1		21	5.29**	[2.22,12.62]	16	1.08	[0.36,3.23]
GT+TT	17	2.62*	[1.02,6.73]	20	0.27*	[0.09,0.81]	25	0.91	[0.24,3.42]
ERCC4 rs1800067									
MN									
GG	29	1		30	1.42	[1.00,2.01]	30	0.76	[0.53,1.09]
GA+AA	11	1.13	[0.73,1.75]	11	0.53	[0.28,1.00]	11	0.96	[0.50,1.85]
CA-chromatid									
GG	29	1		30	2.51**	[1.78,3.54]	30	1.29	[0.90,1.84]
GA+AA	11	1.02	[0.61,1.72]	11	0.49*	[0.25,0.98]	11	0.94	[0.48,1.87]

*P<0.05, **P<0.01

The *wildtype* homozygous or *positive* (in the case of *GSTM1*) genotypes were always the reference category.

CA-total and CA-chromosome mean ratios increased in the *GSTM1 positive* exposed individuals; the increases were more pronounced in the environmentally exposed group. Besides, a significantly higher mean of the CA-chromosome mean ratios was observed in the null control group. Unexpectedly, *GSTM1 null* exposed individuals showed significant decreases in the CA-total and CA-chromosome mean ratios.

A significant increase in the mean %DNAT ratio was observed in the homozygous wild type subjects for *APEX1* polymorphism from the environmentally exposed group, and in *TG* heterozygous individuals from the occupationally exposed group.

CA-total and CA-chromatid mean ratios were significantly increased in the *OGG1* homozygous wild type exposed groups and in the control individuals carrying the *G* variant allele. Also, the same parameters were significantly reduced in occupationally exposed *G* allele carriers.

The *ERCC1* genotypes influenced the %DNAT mean ratio in the environmentally exposed group (*GG* and *GT+TT*), and in the occupationally exposed individuals carrying the T variant allele. Similarly to what has been observed for *GSTM1*, *ERCC1* genotypes showed significant increases of CA-chromosome mean ratio in environmentally exposed homozygous wild type individuals and an opposite trend in controls, where T allele carriers show higher frequencies.

Finally, CA-chromatid mean ratio significantly increased in environmentally exposed wild type homozygotes for *ERCC4* polymorphism, while environmentally exposed individuals with the *A* variant allele showed lower frequencies for CA-chromatid ($P<0.01$) and MN ($P=0.051$).

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V. Discussion

Biological monitoring provides an integrated estimate of exposure by all routes of absorption into the body and evaluates the overall exposure as the sum of different sources of contamination. It gives information on long-term exposure in some cases and helps to assess exposure of an individual within the working environment and the individual factors influencing the pharmacokinetics of the xenobiotic (Maroni, 1983). Nevertheless, when routes of exposure are integrated or combined, environmental monitoring can be helpful either to clarify which route is more significant or to identify the compounds that have to be taken into account in the biological monitoring practice (Maroni, 2000).

Risk assessment should include the measurement of actual impacts on biological endpoints from trace element contamination in soil, surface water, groundwater, air and sediments. By providing risk assessment data, scientific studies may have a strong influence on regulatory policies and on establishing disease prevention strategies.

The results obtained in the environmental geochemical campaigns performed near Panasqueira mine reported a high degree of contamination by several metal(loid)s (Ávila et al., 2008; Grangeia et al., 2011; Salgueiro et al., 2008). Moreover, local health statistics report an elevated number of health complains, with a significant number of individuals with cardiac, respiratory and urinary diseases, and a high rate of deaths by cancer (personal communication).

It was then considered of paramount importance to conduct a study to evaluate the role of environmental contamination in populations living and working nearby, with the following main tasks:

- quantifying the level of several elements - As, Ca, Cd, Cu, Cr, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, S, Se, Si and Zn - in different biological matrices - blood, urine, finger and toe nails and hair (***biomarkers of exposure***);

- evaluating the genotoxic and immunotoxic damage caused by this contamination (***biomarkers of effect***);

- analysing the possible influence of a set of genetic polymorphisms (***biomarkers of susceptibility***).

1. BIOMARKERS OF EXPOSURE

Blood and urine are still the specimens of choice to be analyzed in biomonitoring studies. The levels of most elements have been extensively studied in these matrices and numerous validated biomarkers of exposure are available. Concerning nails and hair samples, in recent years a number of studies using these matrices have been carried out bringing new and improved information that can help to quantify elements in these matrices and validate this approach to measure exposure in the near future.

In biomonitoring of environmental and occupational exposure to toxic elements, nails are generally preferred to hair, as they do not become so easily contaminated (Button et al., 2009). Further, toenails may be a more reliable sample than fingernails, since the latter come into contact with the atmosphere, metallic objects and other substances containing trace elements such as dyes. In contrast, toenails most of the time are hidden in shoes and therefore they have lower contact with trace elements.

Up to now few studies have been conducted comparing the levels of diverse elements in all five different matrices (He, 2011). The information provided by each of them is rather different as blood and urine generally reflect recent exposures (days/few weeks), and hair and nails, particularly toenails, reflect exposures occurring in the last weeks/months. This distinction may be not straightforward for some elements, such as Cd and Pb which accumulate in the human body for years (half life of 10 – 12 years) (Dorne et al., 2011). The combination of results from all the matrices will allow better characterization of the exposure.

Taking into account all this information, our results point to different types of exposure (past/recent) for different elements in the exposed groups (**Table 8**). The group exposed to environmental toxins only apparently experienced a pronounced and continuous (past and recent) exposure to As, a moderate but continuous exposure to Mg, Mn and Zn, a recent exposure to Mo and past exposure to Cr, Ni and S. Since Mo was only analysed in blood samples little can be said about the timeframe of exposure to this compound. The second group (occupationally exposed) experienced a continuous exposure to Zn, recent exposure to Se, and long standing exposure to As, Mn and Pb.

1.1 Comparison with reference ranges

For some of the elements studied the concentrations reported here for blood and urine were above the published reference ranges (**Table 7**). These reference ranges were obtained from samples collected from Italian and Swedish healthy volunteers without a detailed description of the environmental or occupational exposure therefore are only indicative and may change significantly from population to population. Numerous factors such as site of residence, gender, age, diet, lifestyle or geochemical environment need to be taken into account when establishing the reference ranges for a population (Rodushkin et al., 2000), and any comparison must be interpreted carefully. Regarding the Portuguese population, there are few studies that report metal(loid)s levels in exposed populations, and they show wide changes in levels within the same population in different geographic areas (Coelho et al., 2012). Therefore, in this study we compared the element concentration obtained for the exposed populations with those of the controls, as they were matched for age, gender (only the environmentally exposed group), diet, lifestyle, geochemical environment, residence.

1.2 Correlation between matrices

If exposure occurs continuously significant and positive correlations between the different sample types are expected. As and Hg were the only metals that showed correlations between matrices compatible with the presence of recent and past exposure (**Table 9**). As expected, significant and positive correlations between fingernails and toenails were obtained for most of these elements; significant correlations between finger/toenails and hair, and between blood and urine, were archived for some of them. It is important to highlight the fact that for some of the elements not all the matrices were analysed.

1.3 Effect gender, age and smoking habits

From the results in **Table 7** it can be seen that the concentrations of As, Cr, Mn, and Pb, vary significantly among the three groups, with significant differences in three different media. These results were influenced by confounding variables such as gender, age, smoking habits, and factors directly associated to exposure, such as agriculture practice, fish consumption and source of water for consumption. When adjusting for all these variables some of the elements were no longer significantly different between groups (**Table 7** vs. **Table 8**).

Modifying factors, such as gender, age, socioeconomic status, and lifestyle factors in exposure and susceptibility to metal(loid)s have been reported in the literature to play a role in modulating exposure to metal(loid)s. However, most of these parameters are often overlooked and additional studies need to be performed in order to fill gaps. Identification of these gaps provides information on susceptibility factors which is critical to design guidelines for preventive measures (Berglund et al., 2011).

Regarding the effect of gender on the metals levels in the different matrices, our results show that females have higher concentrations of As, Cr, Mg, Mn and Se in urine, fingernails and toenails, while males have higher levels of Fe, Hg, Mg, Pb, S and Zn in blood and hair samples (**Table 10**). The only exception was Mg in hair which was presented with significantly higher concentrations in females. Interestingly, this difference was reported before (Chojnacka et al., 2006; Nowak and Kozlowski, 1998; Quereshi, 1982; Takeuchi et al., 1982). The higher levels of Pb in blood in males is also known and reported in several studies. Milman et al. (1994) suggested that the difference in Pb blood levels between females and males could be explained by the higher content of haemoglobin in men.

Gender differences in the exposure to toxic metals are well documented. In a review paper by Vahter et al. (2007), several references to studies describing significant differences in internal levels of elements between males and females were described and commented. Berglund et al. (2011) reported that females seemed to be more at risk for toxic metal exposure than males, and this founding was confirmed in our study, where females had significantly higher levels of several genotoxic elements, i.e. As, Cr, and Mn. Differences between genders can be due to different patterns of exposure, with one of the genders being more exposed to certain metal(loid)s, although the presence of different toxicokinetic mechanisms between both genders should be taken into account.

Concerning the effect of age, we found older individuals having higher concentrations in blood and urine (As, Cu, Mg, and Zn), and younger individuals having higher concentrations in nails and hair (As, Cr, Fe, Mg, Mn, and Se) (**Table 10**). These differences, besides different patterns of exposure, may be due to different toxicokinetic rates, possibly because of the lower proportion of water in the organism, lower absorption and excretion rates, and possible nutritional deficiencies, and comorbidities in older individuals.

Finally, the analysis of the effect of smoking showed as smokers have significantly higher concentration of several elements, except for Hg in hair that was higher in non-smokers

(**Table 10**). Since tobacco smoke contains Hg, this result was not expected; nevertheless it was also obtained in other studies, specifically in the one published in 2006 by Chojnacka et al.

2. BIOMARKERS OF EFFECT

2.1. Biomarkers of genotoxicity

To understand the mechanism leading to genotoxic effects, several features have to be taken into account, such as the source of exposure (environmental/occupational), the information given by each biomarker in terms of time frame of exposure, and type of damage, and the specific metal(oid) evaluated. The increase in TCR-Mf appears to be associated with occupational exposure, the higher DNA damage (comet assay) with both sources but more intensely with the environmental exposure, while the higher frequency of MN (only in females) and CA seems to be related to environmental exposure (**Table 14**).

Each biomarker reflects different time frame of exposure. Cytogenetic alterations detected by CA and MN reflect exposures that may have been experienced in the few months before sample collection, although exposure in the previous 2-3 years or even in a much longer period may affect these biomarkers (Liu et al., 2010). Mutations detected by the TCR mutation assay reflect exposures experienced during a period of months to 2-3 years before sampling (Taooka et al., 2006). The comet assay provides information on more recent exposures (few hours/days) and reflects a type of damage that is easily repairable.

2.1.1 Effect of metal(loid) concentration

As described in the previous section (biomarkers of exposure) the two exposed groups have different types of exposure. The environmentally exposed group experienced a pronounced and continuous (past and recent) exposure to As, a moderate but continuous exposure to Mg, Mn and Zn, a recent exposure to Mo and past exposure to Cr, Ni and S. The occupationally exposed group experienced a continuous exposure to Zn, recent exposure to Se, and long standing exposure to As, Mn and Pb. Additionally when we evaluated the genotoxic effect of metal(loid) concentration according to their tertile distribution, significant results were obtained for As, Mn and Pb for the majority of the matrices, particularly toenails which presented several significant differences in the two higher tertiles (**Table 15**).

Considering all the previous factors, we can say that our results seem to point to environmental exposure to As (mainly) and Mn as the main conditions inducing genotoxic damage at short term (high levels of DNA damage - %DNAT) and medium-long term

(increased frequencies of MN and CA). Similarly - although with a lower degree of evidence - occupational exposure to As, Mn, and Pb appeared to induce genotoxic damage at short term (high %DNAT), and at medium term (increased TCR-Mf). The increase in TCR-Mf seems to be associated with exposure to Pb as significant increases in the mutation frequency were observed in the second and third tertiles for blood and hair Pb contents, and in the highest tertile for toenails (P=0.060). Garcia-Lestón and co-workers have an extensive work on this field and they also found a significant increase in TCR-Mf due to occupational exposure to Pb (García-Lestón et al., 2010, 2011, 2012b).

2.1.2 Effect age and gender

The major effects of the confounding factors were observed for age in the TCR-Mf and in the %DNAT, with significantly higher levels in older groups, and for gender in the MN frequency, with significantly higher frequencies in females (**Table 12** and **13**). The effect of age on TCR-Mf has been previously described. Akiyama et al. (1995) found a significant increase in TCR-Mf associated with age (increasing 2×10^{-5} for every 10 years of age). The rise in mutation frequency is due to errors associated with DNA replication in each cell division, so the mutation frequency increases in proportion to the number of cell divisions and, in addition, the efficiency of the DNA repair processes decreases with age. Concerning the influence of age on the levels of DNA damage quantified by comet assay there are also some reports, including a major revision made by Möller (2006).

There are several studies reporting an association between gender and cytogenetic damage detected by MN frequency. Bonassi et al. (1995) found that the MN frequencies in females were 20–30% higher than males. This finding may be attributed to preferential aneugenic events involving the X chromosome (Barale et al., 1998; Surrallés et al., 1996). To take into account the potential effect of these parameters on the association between exposure and genotoxic damage all regression models used in this analysis included age and gender as potential confounder or effect modifier.

2.2 Immune markers

Lymphocytes are the primary cells involved in acquired immunity and their number may vary from 20 to 40% of the total cells in human blood. They are highly specialized cells that interact with other cells to initiate immune response. The specificity of the receptor

and functional heterogeneity allows them to respond to virtually any antigen (Descotes, 2004; Tryphonas et al., 2005).

The importance of assessing changes in lymphocyte subsets is related to the existing evidence linking these changes to exposure to immunotoxic compounds and in turn with alterations of the immune response (Biró et al., 2002; Boscolo et al., 1999, Oh et al., 2005; Tulinska et al., 2004). Hernandez-Castro et al. (2009) demonstrated that an increased, diminished, or absent function of immune-system cells is clearly associated with autoimmune diseases, deregulation of the immune response, and defective immune response against neoplastic cells or different pathogens.

2.2.1 Effect of metal(loid) concentration

Significant modifications of immunotoxicity parameters were observed in the exposed groups, specifically in the percentages of lymphocytes subsets (except for %CD19$^+$) (**Table 16**). The effect of exposure on the different lymphocyte subsets varied according to the source of exposure: the environmentally exposed group showed alterations in %CD8$^+$ and CD4$^+$/CD8$^+$, while mine workers in %CD3$^+$, %CD4$^+$ and %CD16$^+$56$^+$ (**Table 18**).

As described in the former section (biomarkers of exposure), the environmentally exposed group experienced a pronounced and continuous (past and recent) exposure to As, a moderate but continuous exposure to Mg, Mn and Zn, a recent exposure to Mo and past exposure to Cr, Ni and S. On the contrary, the occupationally exposed group experienced a continuous exposure to Zn, recent exposure to Se, and long standing exposure to As, Mn and Pb.

When we evaluated the immunotoxic effects in the study individuals according to the tertile distribution of metal(loid) concentrations in their biological samples, significant results were obtained for Pb in toenails (**Table 20**) and Mn in blood (**Table 21**). Considering all this information, our results seem to point to occupational exposure to Pb and environmental and occupational exposure to Mn and as the main factors inducing immunotoxic effects in the study populations.

Exposure to Pb has been shown to adversely affect several immune functions (Chen et al., 2004; Luebke et al., 2006), although the exact mechanism by which this happens is still unclear (García-Lestón et al., 2012b). In our study high levels of lead (2nd and 3rd tertile) in toenails increased the mean ratios of neopterin, kynurenine and Kyn/Trp. Nevertheless, when exposed population was compared to controls these parameters did not show

significant modifications, so it seems that other factors, different from the place of living or the occupation, influence the plasma concentration of neopterin and kynurenine. Different results were obtained by García-Lestón (2012b), who found significantly higher concentrations of tryptophan and significantly lower levels of Kyn/Trp, with no alteration in neopterin levels, in individuals occupationally exposed to Pb. These latter findings concerning neopterin concentrations were confirmed by Engin et al. (2005) in a population with similar exposure. However, it should be taken into account that in the current study exposure was not restricted to Pb but a complex mixture of metal(loid)s had to be considered.

Neopterin is produced by monocyte-derived macrophages as a result of interferon-y (IFNγ) stimulation within the activation of cell-mediated immune response (Weiss et al., 1999). The degradation of tryptophan to form kynurenine is also promoted by IFNγ (Taylor and Feng, 1991). Accelerated tryptophan breakdown, and consequently elevated Kyn/Trp in serum/plasma, has been shown to strongly correlate with neopterin concentrations (Schroecksnadel et al., 2006). This mechanism was confirmed in our study since significant correlations were obtained between these parameters, although only minor increases were found in the exposed groups vs. non-exposed (**Table 16**). Furthermore, our results seem to confirm the presence of an inflammatory background of these metabolic alterations, arguing against a primary role of the liver enzyme tryptophan pyrrolase. In humans, an increased formation of neopterin and enhanced breakdown of tryptophan have been found in viral infections, malignant disorders and autoimmune diseases (Schroecksnadel et al., 2006). Besides, neopterin and Kyn/Trp concentrations provide significant predictive information in patients with various diseases like cardiovascular disease (Pedersen et al., 2011), several forms of cancer (Sucher et al., 2010) and/or with infections and sepsis (Schroecksnadel et al., 2006).

The influence of Mn on the immune system is still unclear, as confirmed by the conflicting results reported in the literature; some studies in animals and human populations demonstrated immunotoxic effects (Antonini et. al., 2012; Nakata et al., 2006), not confirmed by others (Yuan et al., 2006). Our results are in agreement with those obtained by Nakata et al. (2006) who observed a significant decrease in %CD8^{+} and %CD19^{+} associated with exposure to Mn fumes.

2.2.2 Effect age and gender

Immunological biomarkers analyzed in the present study appeared to be influenced by host factors, such as age and gender (**Table 17**, **18** and **19**).

The effect of age in the immune system has been described in several reports (Knight, 2000; Schindowski et al., 2002; Wick et al., 1997). Aging is associated with a defective function of the immune system, which can lead to infections, autoimmune diseases, and cardiovascular or neurodegenerative disorders (Frick et al., 2004). In our study population older individuals presented increased levels of neopterin, kynurenine and Kyn/Trp. Similar results were published by several groups (Diamondstone et al., 1994; Frick et al., 2004; García-Lestón et al., 2012b; Spencer et al., 2010). Increased neopterin concentrations and Kyn/Trp in older healthy people were suggested to be associated with immune activation (Frick et al., 2004). We also found older individuals having lower levels of %CD3$^+$, %CD8$^+$ and %CD19$^+$, similarly to many other publications on similar topics (Chng et al., 2004; Coelho et al., 2011b, 2012; Jentsch-Ullrich et al., 2005; Sansoni et al., 1993; Shahaboddin et al., 1998). The robust consistency of results from the Panasqueira study concerning the effect of aging with evidence existing in the literature provides an intrinsic validation of our results.

The significant influence of gender on %CD19$^+$ (in our study this effect is combined with exposure) was also described in other studies, while females generally presenting higher values (Abdullah et al., 2012; Chng et al., 2004). Remarkably, the variations in the immune system related to age and gender has been linked to several diseases such as autoimmune disorders in females and leukaemia or lymphoma in males and elderly individuals (Jentsch-Ullrich et al., 2005).

2.3 Symptomatology

From all the clinical symptoms evaluated in the exposed populations, cough with expectoration and taking medicines were significantly different among study populations (**Table 22**). The significant decrease observed in these symptoms in the occupationally exposed group may be explained by the "Healthy Worker Effect". According to this concept, working individuals have a lower morbidity when compared to the rest of the population as healthier individuals are more likely to get employment and remain employed (Thygesen et al., 2011). As regards the decrease in taking medicines in the occupationally exposed group, this unexpected finding can be related to a higher

prevalence of males in this group; usually females go to the doctor and take medicines more frequently than males.

3. BIOMARKERS OF SUSCEPTIBILITY

3.1 Polymorphisms in genes involved in the metabolism

Polymorphic genes involved in the metabolism of xenobiotics have been studied in recent decades in order to understand the possible modulator effect of these genetic determinants in genetic damage. Despite the vast number of studies where associations between polymorphic enzymes and individual cancer susceptibility have been established, few studies to evaluate the effect of genetic polymorphisms on genetic damage caused by both environmental and occupational exposure to metal(loid)s have been performed.

In the present study we found a significant influence of *GSTM1* on the effect of exposure to genotoxic agents (**Table 24**). This polymorphic gene has been extensively studied, as individuals presenting the null genotype have a decreased ability to detoxify carcinogens, thus having a higher sensitivity to genetic damage and an increased cancer risk (Rossi et al. 2009). Accordingly, our results showed among the control group a higher CA-chromosome mean ratio in the *GSTM1 null* individuals. On the other hand, the effect of this genotype on the presence of exposure was opposite, i.e. lower CA-total and CA-chromosome mean ratios. Two of the major studies which evaluated the influence of *GSTM1* deletion on CA frequencies were performed by Rossi et al. (2009) and Skjelbred et al. (2010), and they did not find any significant effects.

3.2 Polymorphisms in genes involved in DNA repair

DNA repair mechanisms are vital responses to multiple types of DNA damage, specifically those from exposure to environmental and endogenous carcinogens (McWilliams et al., 2008). Genetic variations in DNA repair genes may modulate DNA repair capacity and, therefore influence risk for development of cancer and other mutation related diseases (Kiyohara and Yoshimasu, 2007).

As regards the DNA repair genotypes analyzed, significant influences were found for *APEX1* rs1130409, *OGG1* rs1052133, *ERCC1* rs3212986, and *ERCC4* rs1800067 (**Table 24**).

The *APEX1* gene encodes the major apurinic/apirimidinic (AP) endonuclease and has a major role in the BER of DNA damage (Shen et al., 2005). A significant increase in the DNA damage mean ratio was found in both exposed groups in individuals carrying the *T* allele. This result is in agreement with evidence supporting an association between the *G*

allele and lower levels of DNA damage with a consequent decreased risk of a number of human cancers (Yu et al., 2012).

The human *OGG1* gene encodes a protein responsible for excision of 8-oxoGua, the main base lesion caused by oxidative damage to DNA. If the lesion is not repaired, it can pair with adenine leading to a *GC* to *TA* transversion. Significant influence of exposure on CA-total and CA-chromatid was observed in all exposed individuals wild type homozygous, as well as in the controls carriers of the variant *G* allele. The vast majority of studies report a lower ability of the *G* allele to repair DNA damage and consequently this genotype should prevent mutagenic events (Bravard et al., 2009; Yamane et al., 2004). Our results showed also combined effect of exposure and genotype in occupationally exposed people carrying the variant *G* allele, although these findings go in the opposite direction i.e. decreases in the mean ratios. With regard to this result, it has been suggested that the effect of this polymorphism on DNA repair capacity may differ with the type and extent of exposure and can be influenced by the interaction with other genetic polymorphisms (Mateuca et al. , 2008).

The excision repair cross-complementing (*ERCC*) gene family reduces damage to DNA via NER. The *ERCC1* gene encodes a protein that, along with *ERCC4*, functions in a complex involved in the 5' incision made during NER process (van Vuuren et al., 1995). Our results for this polymorphism showed significant combined effect with exposure in both exposed groups concerning %DNAT (increases in the mean ratio), and only in the environmentally exposed group for CA-chromosome (decrease in the mean ratio). These contrasting results might be in part explained by the different genotoxic endpoint detected by these assays. The number of reports analysing the effect of this polymorphism is scarce. Costa et al. (2008) did not find a significant influence in any of the genotoxicity biomarkers studied, whereas Zienolddiny et al. (2006) found *T* allele to be less frequent in non-small cell lung cancer cases with higher PAH-DNA adduct levels, and speculated that individuals presenting the *T* allele may have suboptimal DNA repair capacity. In agreement with this report the result of this study showed an increase in the CA-chromosome mean ratio in the control individuals carrying the *T* allele.

Finally, the influence of ERCC4 polymorphism on the frequency of MN and CA-chromatid seems to be due to the protective effect of the *A* allele (lower frequencies) in the environmental exposed group. Also for this polymorphism few reports have been published. No effect was observed by Costa et al. (2008) on MN test, SCE or comet assay results. In addition, Park et al. (2002) reported no significant relationship between *ERCC4*

and lung cancer. Nevertheless, significant association between the *A* allele and breast cancer risk was reported by Smith et al. (2003), particularly when in combination with the variants alleles of XRCC1 (rs1799782 and rs25487) and XRCC3 (rs861539).

V. Conclusions

Overall our results are in agreement with those obtained in the previous environmental studies performed in Panasqueira Mine area, showing that populations living nearby and working in the mine are exposed to several metal(loid)s originated by mining activities. The most significant exposure was to As, particularly in individuals exposed through the environment. Exposure to other elements such as Cr, Mn, Ni, Pb, Se and Zn also occurs but not at the same extent (amount of element/time of exposure). Our results indicate that the environmentally exposed group is more affected, specifically females, as they presented significantly higher values of the most toxic elements, i.e., As, Cr, Mn and Ni.

Our results also show major effects of host factors and smoking habits on the levels of metal(loid)s in the different biological samples analysed, strongly influencing the results obtained. Studies with a higher number of individuals, analysing all the elements in all the matrices need to be performed in order to better characterize the factors influencing the exposure, the toxicokinetic processes in the population groups, and the feasibility of the different biological samples for exposure assessment.

Results also point to increased genotoxic damage and immunotoxicity experienced by populations environmentally and occupationally exposed to metal(loid)s contamination derived from the Panasqueira mine activities. To strengthen the absence of causal association between mine activities and this damage, the level of most biomarkers of exposure was quantitatively associated with the intensity of genotoxic and immunotoxic damage.

The extent of genetic damage associated to exposure to metal(loid)s was modulated by some of the selected genetic polymorphisms of enzymes involved in the metabolism of metal(loid)s and DNA repair processes. Still these data are difficult to interpret and definitive conclusions on their influence cannot be reached.

In conclusion, the presence of genotoxic damage and immunotoxicity in exposed populations, their consistency with individual data of exposure, and the identification of sub-groups of susceptible populations pose a public threat that may result in an increased risk of developing cancer and other diseases. Competent authorities are urged to intervene in this area and implementing preventive policies aimed to help protecting exposed populations. Not only human populations are at risk but the entire ecosystem.

Results from this study are of paramount importance not only for these particular populations but to others exposed to similar conditions. A greater understanding of the relationship between exposure, individual susceptibility and biological effect, enables the development of preventive measures, leading to a reduction in cancer risks for humans

exposed to similar conditions. It is crucial to conduct regular monitoring and surveillance activities of the environment, miners and community's health. The combination of environmental and human biomonitoring studies may synergistically increase the knowledge about the bioaccessibility and bioavailability of toxic elements, which is essential to assess the potential risks to human health.

VII. REFERENCES

Abbas, A.K., and Lichtman, A.H. 2004. Células y tejidos del sistema inmunitario. In *Inmunología celular y molecular*. 5ª edición. Elsevier. Madrid. pp. 16-39.

Abdullah, M., Chai, P.S., Chong, M.Y., Tohit, E.R., Ramasamy, R., Pei, C.P., and Vidyadaran, S. 2012. Gender effect on in vitro lymphocyte subset levels of healthy individuals. *Cell Immunol.* 272: 214-219.

Akiyama, M., Kyoizumi, S., Hirai, Y., Kusunoki, Y., Iwamoto, K. S., and Nakamura, N. 1995. Mutation frequency in human blood cells increases with age. *Mutat. Res.* 338: 141-149.

Albertini, R.J., Anderson, D., Douglas, G.R., Hagmar, L., Hemminki, K., Merlo, F., Natarajan, A.T., Norppa, H., Shuker, D.E., Tice, R., Waters, M.D., and Aitio, A. 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety. *Mutat Res.* 463(2):111-172.

Albertini, R., Bird, M., Doerrer, N., Needham, L., Robinson, S., Sheldon, L., and Zenick, H. 2006. The use of biomonitoring data in exposure and human health risk assessment. *Environ. Health Perspect.* 114: 1755-1762.

Alimonti, A., Bocca, B., Mannella, E., Petrucci, F., Zennaro, F., Cotichini, R., D'Ippolito, C., Agresti, A., Caimi, S., and Forte, G. 2005. Assessment of reference values for selected elements in a healthy urban population. *Ann. Ist. Super Sanita* 41(2): 181-187.

Amorim L. 2003. Os biomarcadores e a sua aplicação na avaliação da exposição aos agentes químicos ambientais. *Ver. Bras. Epidemiol.* 6(1): 158-170.

Andonova IE, Justenhoven C, Winter S, Hamann U, Baisch C, Rabstein S, Spickenheuer A, Harth V, Pesch B, Brüning T, Ko YD, Ganev V, and Brauch H. 2010. No evidence for glutathione S-transferases GSTA2, GSTM2, GSTO1, GSTO2, and GSTZ1 in breast cancer risk. *Breast Cancer Res. Treat.* 121(2):497-502.

Antonini, J.M., Zeidler-Erdely, P.C., Young, S.H., Roberts, J.R., and Erdely, A. 2012. Systemic immune cell response in rats after pulmonary exposure to manganese-containing particles collected from welding aerosols. *J. Immunotoxicol.* 9: 184-192.

Ataian, Y., and Krebs, J.E. 2006. Five repair pathways in one context: chromatin modification during DNA repair. *Biochem. Cell Biol.* 84: 490-504.

Ávila, P., Silva, E., Salgueiro, A., and Farinha, J. 2008. Geochemistry and mineralogy of mill tailings impoundments from the Panasqueira mine (Portugal): Implications for the surrounding environment. *Mine Water and the Environ.* 27(4): 210-224.

Au, W.W. 2007. Usefulness of biomarkers in population studies: From exposure to susceptibility and to prediction of cancer. *Int. J. Hyg. Environ. Health* 10: 239-246.

Bal, W., Protas, A.M., and Kasprzak, K.S. 2011. Genotoxicity of metal ions: chemical insights. *Met. Ions Life Sci.* 8: 319-373.

Balmain, A., Gray, J., and Ponder, B. A. 2003. The genetics and genomics of cancer. *Nat. Genet.* 33: 238-44.

Barale, R., Chelotti, L., Davini, T., Del Ry, S., Andreassi, M.G., Ballardin, M., Bulleri, M., He, J., Baldacci, S., Di Pede, F., Gemignani, F., and Landi, S. 1998. Sister chromatid

exchange and micronucleus frequency in human lymphocytes of 1,650 subjects in an Italian population: II. Contribution of sex, age, and lifestyle. *Environ. Mol. Mutagen.* 31(3): 228-242.

Bencjo, V. 1995. Use of human hair as a biomarker in the assessment of exposure to pollutants in occupational and environmental settings. *Toxicology* 101:29–39.

Berglund, M., Lindberg, A., Rahman, M., Yunus, M., Grandér, M., Lönnerdal, B., and Vahter, M. 2011. Gender and age differences in mixed metal exposure and urinary excretion. *Environ Res.* 111(8): 1271-1279.

Bernstein, B.E., Birney, E., Dunham, I., Green, E.D., Gunter, C., and Snyder, M. 2012. An integrated encyclopedia of DNA elements in the human genome. *Nature* 489: 57-74.

Bessho, T., Roy, R., Yamamoto, K., Kasai, H., Nishimura, S.,Tano, K., and Mitra, S. 1993. *Proc. Natl. Acad. Sci. USA* 90: 8901-8904.

Biró, A., Pállinger, E., Major, J., Jakab, M. G., Klupp, T., Falus, A., and Tompa, A. 2002. Lymphocyte phenotype analysis and chromosome aberration frequency of workers occupationally exposed to styrene, benzene, polycyclic aromatic hydrocarbons or mixed solvents. *Immunol. Lett.* 81: 133-140.

Blank, J.A., Luster, M.I., Langone, J.J., and Wilson, S.D. 2000. Immunotoxicology - Regulatory and Risk Assessment Concepts. *Int. J. Toxicol.* 19: 95-106.

Bloot, C., and de Wolf, L.C.M. 1953. Geological features of the Panasqueira tin-tungsten ore occurrence (Portugal). *Bol. Soc. Geol. Port.* 11(1): 1-58.

Bolognesi, C. 2003. Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutat. Res.* 543(3): 251-272.

Bonassi, S., and Au, W. 2002. Biomarkers in molecular epidemiology studies for health risk prediction. *Mutat. Res.* 511: 73-86.

Bonassi. S., Bolognesi, C., Abbondandolo, A., Barale, R., Bigatti, P., Camurri, L., Dalpra, L., De Ferrari, M., Forni, A., and Lando, C. 1995. Infuence of sex on cytogenetic end points: Evidence from a large human sample and review of literature. *Cancer Epidemiol. Biomarkers Prev.* 4: 671–679.

Bonassi, S., Ugolini, D., Kirsch-Volders, M., Stromberg, U., Vermeulen, R., and Tucker, J.D. 2005. Human population studies with cytogenetic biomarkers: review of the literature and future prospective. *Environ. Mol. Mutagen.* 45: 258-270.

Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., and Holland, N. 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis* 28:625-631.

Bortoli, A., Gerotto, M., Marchiori, M., Palonta, R., and Troncon, A. 1992. Applications of inductively coupled plasma mass spectrometry to the early detection of potentially toxic elements. *Microchem. J.* 46: 167-173.

Boscolo, P., Di Gioacchino, M., Sabbioni, E., Benvenuti, F., Conti, P., Reale, M., Bavazzano, P., and Giuliano, G. 1999. Expression of lymphocyte subpopulations,

cytokine serum levels, and blood and urinary trace elements in asymptomatic atopic men exposed to an urban environment. *Int. Arch. Occup. Environ. Health* 72: 26-32.

Bravard, A., Vacher, M., Moritz, E., Vaslin, L., Hall, J., Epe, B., and Radicella, J.P. 2009. Oxidation status of human OGG1-S326C polymorphic variant determines cellular DNA repair capacity. *Cancer Res.* 69(8): 3642-3649.

Breiter, K. 2001. *Report about Laboratory Investigations of Rock Samples from the Panasqueira Mine and Recommendations for Future Exploration, Novembro 2001*.

Brima, E., Haris, P., Jenkins, R., Polya, D., Gault, A., and Harrington, C. 2006. Understanding arsenic metabolism through a comperative study of arsenic levels in the urine, hair and fingernails of healthy volunteers from three unexposed ethnic groups in the United Kingdom. *Toxicol. Appl. Pharmacol.* 216: 122-130.

Brookes, A.J. 1999. The essence of SNPs . *Gene* 234(2): 177-186.

Button, M., Jenkin, G., Harrington, C., and Watts, M. 2009. Human toenails as a biomarker of exposure to elevated environmental arsenic. *J. Environ. Monit.* 11: 610-617.

Cabassi, E. 2007. The immune system and exposure to xenobiotics in animals. *Vet. Res. Commun.* 31: 115-120.

Candeias, C. 2010. *Modelação do impacted a Mina da Panasqueira nos ecossistemas e saúde humana – uma abordagem multidisciplinar*. Projecto de Dissertação – Programa Doutoral em Geociências. Departamento de Geociências, Universidade de Aveiro.

Cao, Q., Qin, C., Meng, X., Ju, X., Ding, Q., Wang, M., Zhu, J., Wang, W., Li, P., Chen, J., Zhang, Z., and Yin, C. 2011. Genetic polymorphisms in APE1 are associated with renal cell carcinoma risk in a Chinese population. *Mol. Carcinog.* 50: 863-870.

Capuron, L., Moranis, A., Combe, N., Cousson-Gelie, F., Fuchs, D., De Smedt- Peyrusse, V., Barberger-Gateau, P., and Layé, S. 2009. Vitamin E status and quality of life in the elderly: Influence of inflammatory processes. *Br. J. Nutr.* 102: 1390-1394.

Capuron, L., Schroecksnadel, S., Féart, C., Aubert, A., Higueret, D., Barberger-Gateau, P., Layé, S., and Fuchs, D. 2011. Chrinic low-grade inflammation in elderly persons is associated with altered tryptophan and tyrosine metabolism: role in neuropsychiatric symptoms. *Biol. Psychiatry* 70: 175-182.

Caroli, S., Senofonte, O., Violante, N., Fornarelli, L., and Powar, A. 1992. Assessment of reference values for elements in hair of urban normal subjects. *Michrochem J.* 46: 174-183.

Carrano, A., and Nataranjan, A. 1988. Considerations for population monitoring using cytogenetic techniques. *Mutat. Res.* 204: 379-406.

Cavey, G., and Gunning, D. 2006. Updated technical report on the Panasqueira mine, Distrito de Castelo Branco, Portugal. *OREQUEST*. pp. 67.

Chatt, A., Secord, C., Tiefenbach, B., and Jervis, R. 1980. Scalp hair as a monitor of community exposure to environmental pollutants. In *Hair, trace elements and human illness* edited by A. C. Brown and R. Crounse. Praeger.

Chen, S., Golemboski, K.A., Piepenbrink, M., and Dietert, R.R. 2004. Developmental immunotoxicity of lead in the rat: Influence of maternal diet. *J. Toxicol. Environ. Health A* 67: 495–511.

Chen, Y., and Guillemin, G.J. 2009. Kynurenine pathway metabolites in humans: disease and healthy States. *Int. J. Tryptophan Res.* 2: 1-19.

Chng, W.C., Tan, G.B., and Kuperan, P. 2004. Establishment of adult peripheral blood lymphocyte subset reference range for an Asian population by single platform flow cytometry: Influence of age, sex and race and comparison with other published studies. *Clin. Diagn. Lab. Immunol.* 11: 168-173.

Chojnacka, K., Górecka, H., and Górecki, H. 2006. The effect of age, sex, smoking habit and hair color on the composition of hair. *Environ. Toxicol. Pharmacol.* 22: 52-57.

Christmann, M., Tomicic, M.T., Roos, W.P., and Kaina, B. 2003. Mechanisms of human DNA repair: an update. *Toxicology* 193(1-2): 3-34.

Cleaver, J.E., Thompson, L.H., Richardson, A.S., and States, J.C. 1999. A summary of mutations in the UV-sensitive disorders: xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy. *Hum. Mutat.* 14(1): 9-22.

Clevers, H., Dunlap, S., and Terhorst, C. 1988. The transmembrane orientation of the epsilon chain of the TcR/CD3 complex. *Eur. J. Immunol.* 18(5): 705-710.

Coelho, P., Costa, S., Silva, S., Walter, A., Ranville, J., Sousa, A.C., Costa, C., Coelho, M., García-Lestón, J., Pastorinho, M.R., Laffon, B., Pásaro, E., Harrington, C., Taylor, A., and Teixeira, J.P. 2012. Metal(loid)s levels in biological matrices from human populations exposed to mining contamination – Panasqueira Mine (Portugal). *J. Toxicol. Environ. Health A* 75: 893-908.

Coelho, P., García-Lestón, J., Silva, S., Costa, C., Costa, S., Coelho, M., Laffon, B., Pásaro, E., and Teixeira, J.P. 2011b. Geno- and Immunotoxic effects on populations living near a mine - Case study: Panasqueira mine. *J. Toxicol. Environ. Health A* 74: 1076-1086.

Coelho, P., Silva, S., Roma-Torres, J., Costa, C., Henriques, A., Teixeira, J., Gomes, M., and Mayan, O. 2007. Health impact of living near an abandoned mine—Case study: Jales Mines. *Int. J. Hyg. Environ. Health* 210: 399-402.

Coelho, P., Teixeira, J., and Gonçalves, O. 2011a. Mining activities: Health impacts. In *Encyclopedia of Environmental Health* edited by O. Nriagu, vol. 3. Burlington: Elsevier. pp: 788–802.

Coles, B.F., and Kadlubar, F.F. 2005. Human alpha class glutathione S-transferases: genetic polymorphism, expression, and susceptibility to disease. *Methods Enzymol.* 401: 9-42.

Collins, A.R. 2004. The Comet Assay for DNA damage and repair. Principles, Applications, and Limitations. *Mol.Biotechnol.* 26: 249-261.

Conde, J., Silva, S.N., Azevedo, A.P., Teixeira, V., Pina, J.E., Rueff, J., and Gaspar, J. 2009. Association of common variants in mismatch repair genes and breast cancer susceptibility: a multigene study. *BMC Cancer* 9: 344.

Conde, L.N., Pereira, V., Ribeiro, A. and Tadeu, D. 1971. *Jazigos hipogénicos de estanho e volfrâmio. Livro-guia da Excursão nº 7, I Congresso Hispano-Luso-Americano de Geologia Económica, Lisboa-Madrid.* pp. 81.

Correa, A., and Naique, R.A. 1998. *Minas Panasqueira, 100 Years of Mining History paper presented at the 1998 International Tungsten Industry Association (ITIA) conference.*

Corrêa de Sá, A., Naique, R.A., and Nobre, E. 1999 Minas da Panasqueira – 100 anos de História. *Bol. de Minas* 36(1): 3-22.

Costa, C., Teixeira, J.P., Silva, S., Roma-Torres, J., Coelho, P., Gaspar, J., Alves, M., Laffon, B., Rueff, J., and Mayan, O. 2006. Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides. *Mutagenesis* 21: 343-350.

Costa, S., Coelho, P., Costa, C., Silva, S., Mayan, O., Santos, L.S., Gaspar, J., and Teixeira, J.P. 2008. Genotoxic damage in pathology anatomy laboratory workers exposed to formaldehyde. *Toxicology* 252: 40-48.

Creason, J., Hinners, T., Bumgarner, J., and Pinkert, C. 1982 Trace elements in hair, as related to exposure in metropolitan New York. *Clin. Chem.* 21: 603–612.

Delft, J., Baan, R., and Roza, L. 1998. Biological effect markers for exposure to carcinogenic compound and their relevance for risk assessment. Critical Reviews in *Toxicology* 28(5): 477-510.

Delves, H. 1988. Biomedical applications of ICP-MS. *Chem. Britain* 1: 1009-1012.

Descotes, J. 2004. Classification of immunotoxicants. In I*mmunotoxicology of drugs and chemicals: an experimental approach.* Volume I: Principles and methods of immunotoxicology. Amsterdam : Elsevier. pp.379-385.

Diamondstone, L.S., Tollerud, D.J., Fuchs, D., Wachter, H., Brown, L.M., Maloney, E., Kurman, C.C., Nelson, D.L., and Blattner, W.A. 1994. Factors influencing serum neopterin and beta 2-microglobulin levels in a healthy diverse population. *J. Clin. Immunol.* 14: 368-74.

D'Orey, F.C. 1967. Tin-tungsten mineralization and paragenesis in the Panasqueira and Vale de Ermida mining districts, Portugal. Comunicações Serviços Geológicos de Portugal, tomo LII, pp: 117-167.

Dorne, J., Kass, G., Borgajandi, L., Amzal, B., Bertelsen, U., Castoldi, A. Heppner, C., Eskola, M., Fabiansson, S., Ferrari, P., Scaravelli, E., Dogliotti, E., Fuerst, P., Boobis, A.R., and Verger, P. 2011. Human risk assessment of heavy metals: principals and applications. In *Metal ions in toxicology: effects, interactions, interdependencies* edited by Sigel A., Sigel H. and Sigel R. K. O. Cambridge, UK: The Royal Society of Chemistry. pp.27-60.

Druyan, M.E., Bass, D., Puchyr, R., Urek, K., Quig, D., Harmon, E., and Marquardt, W. 1998. Determination of reference ranges for elements in human scalp hair. *Biol. Trace Elem. Res.* 62(3): 183-197.

Duell, E.J., Wiencke, J.K., Cheng, T.-J., Varkoni, A., Zuo, Z.F., Ashok, T.D.S., Mark, E.J., Wain, J.C., Christiani, D.C., and Kelsey, K.T. 2000. Polymorphisms in the DNA repair genes XRCC1 and ERCC2 and biomarkers of DNA damage in human blood mononuclear cells. *Carcinogenesis* 21: 965-971.

Duyff, R.L. 2006. *American Dietetic Association Complete Food and Nutrition Guide*, 3rd ed. John Wiley & Sons, Inc.: Hoboken, New Jersey.

e-Ecorisk 2007. *A Regional Enterprise Network Decision-Support System for Environmental Risk and Disaster Management of Large-Scale Industrial Spills. WP3-case study characterization. Deliverable 3.1*. pp: 20.

El-Zein, R.A., Schabath, M.B., Etzel, C.J., Lopez, M.S., Franklin, J.D., and Spitz, M.R. 2006. Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. *Cancer Res.* 66: 6449-6456.

Engin, A.B., Tuzun, D., and Sahin, G. 2006. Evaluation of pteridine metabolism in battery workers chronically exposed to lead. *Hum. Exp. Toxicol.* 25: 353–359.

Farmer, P., Kyrtopoulos, S., and Emeny, J. 2007. *State of validation of biomarkers of carcinogen and early effects and their applicability to molecular epidemiology.* Nofer Institute of Occupational Medicine: Poland.

Fenech, M. 1993. The cytokinesis-block micronucleus technique and its application to genotoxicity studies in human populations. *Environ. Health Perspect.* 101(3):101-107.

Fenech, M. 2000. The in vitro micronucleus technique. *Mutat. Res.* 455(1-2): 81-95.

Fenech, M. 2005. In vitro micronucleus technique to predict chemosensitivity. *Methods Mol. Med.* 111: 3-32.

Fenech, M. 2007. Cytokinesis-block micronucleus cytome assay. *Nat. Protoc.* 2: 1084-104.

Feuk, L., Carson, A.R., and Scherer, S.W. 2006. Structural variation in the human genome. *Nat.Rev.Genet.* 7: 85-97.

Frick, B., Schroecksnadel, K., Neurauter, G., Leblhuber, F., and Fuchs, D. 2004. Increasing production of homocysteine and neopterin and degradation of tryptophan with older age. *Clin. Biochem.* 37: 684-687.

Fuchs, D., Baier-Bitterlich, G., Wede, I., and Wachter, H. 1997. Reactive oxygen and apoptosis. In *Oxidative stress and the molecular biology of antioxidant defenses* edited by Scandalios J. Cold Spring Harbor Press: New York. pp. 139–67.

Fuchs, D., Weiss, G., Reibnegger, G., and Wachter, H. 1992. The role of neopterin as a monitor of cellular immune activation in transplantation, inflammatory, infectious, and malignant diseases. *Crit. Rev. Clin. Lab. Sci.* 29(3-4): 307-41.

Fuchs, D., Weiss, G., and Wachter, H. 1993. Neopterin, biochemistry and clinical use a marker for cellular immune reactions. *Int. Arch. Allergy Immunol.* 101:1-6.

García-Lestón, J. 2012a. *Exposición ocupacional a plomo: efectos genotoxicos e inmunotóxicos.* Tesis Doctoral. Departamento de Biología Celular y Molecular Área de Genética, Universidade da Coruña.

García-Lestón, J., Méndez, J., Pásaro, E., and Laffon, B. 2010. Genotoxic effects of lead: an updated review. *Envir. Int.* 36: 623-36.

García-Lestón, J., Roma-Torres, J., Vilares, M., Pinto, R., Cunha, L., Prista, J., Teixeira, J.P., Mayan, O., Pásaro, E., Méndez, J., and Laffon B. 2011. Biomonitoring of a population of Portuguese workers exposed to lead. *Mutat. Res.* 721: 81-88.

García-Lestón, J., Roma-Torres, J., Vilares, M., Pinto, R., Prista, J., Teixeira, J.P., Mayan, O., Conde, J., Pingarilho, M., Gaspar, J.F., Pásaro, E., Méndez, J., and Laffon, B. 2012b. Genotoxic effects of occupational exposure to lead and influence of polymorphisms in genes involved in lead toxicokinetics and in DNA repair. *Environ. Int.* 43: 29-36.

Garte, S., and Gaspari, L. 2001. Metabolic gene polymorphism frequencies in control populations. *Cancer Epidemiol. Biomarkers Prev.*10: 1239-1248.

Gaspar, J., Faber, A., and Rueff, J. 2006. Variabilidade Genética e susceptibilidade individual para patologia Multifactorial. *Revista Acção Médica* 2: 41-63.

Gaspar, J., Rodrigues, S., Gil, O.M., Manita, I., Ferreira, T.C., Limbert, E., Gonçalves, L., Pina, J.E., and Rueff, J. 2004. Combined effects of glutathione S-transferase polymorphisms and thyroid cancer risk. *Cancer Genet. Cytogenet.* 151: 60-67.

Gemignani, F., Landi, S., Szeszenia-Dabrowska, N., Zaridze, D., Lissowska, J., Rudnai, P., Fabianova, E., Mates, D., Foretova, L., Janout, V., Bencko, V., Gaborieau, V., Gioia-Patricola, L., Bellini, I., Barale, R., Canzian, F., Hall, J., Boffetta, P., Hung, R.J., and Brennan, P. 2007. Development of lung cancer before the age of 50: the role of xenobiotic metabolizing genes. *Carcinogenesis* 28(6): 1287-1293.

Gil, F., and Pla, A. 2001. Biomarkers as Biological Indicators of xenobiotic exposure. *J. Appl. Toxicol.* 21: 245-255.

Gomes, B.C., Silva, S.N., Azevedo, A.P., Manita, I., Gil, O.M., Ferreira, T.C., Limbert, E., Rueff, J., and Gaspar, J.F. 2010. The role of common variants of non-homologous end-joining repair genes XRCC4, LIG4 and Ku80 in thyroid cancer risk. *Oncol. Rep.* 24:1079-1085.

Goode, E.L., Ulrich, C.M., and Potter, J.D. 2002. Polymorphisms in DNA repair genes and asssociations with cancer risk. *Cancer Epidemiol Biomarkers Prev.* 11: 1513-1530.

Goullé, J.P., Mahieu, L., Castermant, J., Neveu, N., Bonneau, L., Lainé, G., Bouige, D., and Lacroix, C. 2005. Metal and metalloid multi-elementary ICP-MS validation in whole blood, plasma, urine and hair. Reference values. *Forensic Sci. Int.* 153(1): 39-44.

Goyer R. 2004. *Issue Paper On The Human Health Effects Of Metals.* U.S. Environmental Protection Agency: Lexington, MA, USA.

Grangeia, C., Ávila, P., Matias, M., and Ferreira da Silva, E. 2011. Mine tailings integrated investigations: The case of Rio tailings (Panasqueira Mine, Central Portugal). *Eng. Geol.* 123: 359-372.

Griess, P. 1879. Bemerkungen zu der Abhandlung der HH. Weselky und Benedikt "Ueber einige Azoverbindungen." *Chemistry* 12: 426–428.

Guikema, J.E., Linehan, E.K., Tsuchimoto, D., Nakabeppu, Y., Strauss, P.R., Stavnezer, J., and Schrader, C.E. 2007. APE1- and APE2-dependent DNA breaks in immunoglobulin class switch recombination. *J. Exp. Med.* 204: 3017-3026.

Hagmar, L., Stromberg, U., Bonassi, S., Hansteen, I.-L., Knudsen, L.E., Lindholm, C., and Norppa, H. 2004. Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts. *Cancer Res.* 64: 2258–2263.

Harries, L.W., Stubbins, M.J., Forman, D., Howard, G.C., and Wolf, C.R. 1997. Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis* 18: 641-644.

Harris, M.J., Coggan, M., Langton, L., Wilson, S.R., and Board, P.G. 1998. Polymorphism of the Pi class glutathione S-transferase in normal populations and cancer patients. *Pharmacogenetics* 8(1): 27-31.

Hartwig, A. 1994. Role of DNA Repair Inhibition in Lead- and Cadmium-Induced Genotoxicity: A Review. *Environ. Health Perspect.* 102: 45-50.

He, K. 2011. Trace elements in nails as biomarkers in clinical research. *Europ. J. Clin.* Inv. 4: 98-102.

Health Canada 2004. *Canadian Handbook on Health Impact Assessment* (4 vols.) A Report of the Federal/Provincial/Territorial Committee on Environmental and Occupational Health. Ottawa: Health Canada.

Heng, H.H., Stevens, J.B., Liu, G., Bremer, S.W., and Ye, C.J. 2004. Imaging genome abnormalities in cancer research. *Cell Chromosome* 3(1): 1.

Henkler, F., Brinkmann, J., and Luch, A. 2010. The role of oxidative stress in carcinogenesis induced by metals and xenobiotics. *Cancers* 2: 376–396.

HERAG05 (Health Risk Assessment Guidance for Metals fact sheet 05). 2007. *Eurometaux-ICMM-Eurofer Fact sheets related to Effects Assessment* – Mutagenicity. Available at: http://www.ebrc.de/industrial-chemicals-reach/projects-and-references/downloads/HERAG_FS_05_August_07.pdf [accessed 16 of September 2012].

Hernandez-Castro, B., Doniz-Padilla, L. M., Salgado-Bustamante, M., Rocha, D., Ortiz-Perez, M. D., Jimenez-Capdeville, M. E., Portales-Pérez, D. P., Quintanar-Stephano, A., and González-Amaro, R. 2009. Effect of arsenic on regulatory T cells. *J. Clin. Immunol.* 29: 461–69.

Hinwood, A., Sim, M., de Klerk, N., Drummer, O., Gerostamoulos, J., and Bastone, E. 2002. Are 24-hour urine samples and creatinine adjustment required for analysis of inorganic arsenic in urine in urine in population studies? *Environ. Res. Sec. A* 88: 219–224.

Hoeijmakers, J.H. 1993. Nucleotide excision repair: II. From yeast to mammals. *Trends Genet.* 9: 211-217.

Huang, R.S., Chen, P., Wisel, S., Duan, S., Zhang, W., Cook, E.H., Das, S., Cox, N.J., and Dolan, M.E. 2009. Population-specific GSTM1 copy number variation. *Hum. Mol. Genet.*18: 366-372.

Jadhav, S.H., Sarkar, S.N., and Tripathit, H.C. 2006. Cytogenetic effects of a mixture of selected metals following subchronic exposure through drinking water in male rats. *Indian J. Exp. Biol.* 44: 997–1005.

Jenny, M., Klieber, M., Zaknun, D., Schroecksnadel, S., Kurz, K., Ledochowski, M., Schennach, H., and Fuchs, D. 2011. In vitro testing for anti-inflammatory properties of compounds employing peripheral blood mononuclear cells freshly isolated from healthy donors. *Inflamm. Res.* 60: 127-135.

Jentsch-Ullrich, K., Koenigsmann, M., Mohren, M., and Franke, A. 2005. Lymphocyte subsets' reference ranges in an ageand gender-balanced population of 100 healthy adults - A monocentric German study. *Clin. Immunol.* 116: 192-197.

Julivert, F., Fontboté, J., Ribeiro, A., and Conde, L. 1974. Mapa *Tectonico de la Península Ibérica y Baleares, escala 1:1,000,000.* Inst. Geol. Minero España, Madrid, pp. 113.

Kanias, G. 1985. Determination of trace elements in eyeshadow face powder and rouge make-up cosmetics by neutron activation analysis. *J. Radioanal. Nucl. Chem.* 89(2): 487–496.

Kassie, F., Parzefall, W., and Knasmuller, S. 2000. Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. *Mutat. Res.* 463:13-31.

Kauppinen, T.M., Chan, W.Y., Suh, S.W., Wiggins, A.K., Huang, E.J., and Swanson, R.A. 2006. Direct phosphorylation and regulation of poly(ADP-ribose) polymerase-1 by extracellular signal-regulated kinases 1/2. *Proc. Natl. Acad. Sci. USA* 103:7136–7141.

Kelly, W.C., and Rye, R.O. 1979. Geologic, fluid inclusion and stable isotope studies of the tin-tungsten deposits of Panasqueira, Portugal. *Econ. Geol.* 74: 1721-1822.

Kirsch-Volders, M., Mateuca, R.A., Roelants, M., Tremp, A., Zeiger, E., Bonassi, S., Holland, N., Chang, W.P., Aka, P.V., DeBoeck, M., Godderis, L., Haufroid, V., Ishikawa, H., Laffon, B., Marcos, R., Migliore, L., Norppa, H., Teixeira, J.P., Zijno, A., and Fenech, M. 2006. The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo. *Cancer Epidemiol. Biomarkers Prev.* 15: 1038–1042.

Kitteringham, N.R., Palmer, L., Owen, A., Lian, L.Y., Jenkins, R., Dowdall, S., Gilmore, I., Park, B.K., and Goldring, C.E. 2007. Detection and biochemical characterisation of a novel polymorphism in the human GSTP1 gene. *Biochim. Biophys. Acta* 1770(8):1240-1247.

Kiyohara, C., and Yoshimasu, K. 2007. Genetic polymorphisms in the nucleotide excision repair pathway and lung cancer risk: a meta-analysis. *Int. J. Med. Sci.* 4: 59–71.

Knight, J.A. 2000. Review: Free radicals, antioxidants and immune system. *Ann. Clin. Lab. Sci.* 30:145–58.

Kronenberg, M., Siu, G., Hood, L. E., and Shastri, N. 1986. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Annu. Rev. Immunol.* 4: 529-591.

Kuangfei, L., Yaling, X., Xuefeng, L., Zouli, W., Bukkens, S., Tommaseo, M., and Paoletti, M. 1999. Metallic elements in hair as a biomarker of human exposure to environmental pollution: A preliminary investigation in Hubei Province. *Crit. Rev. Plant Sci.* 8: 417–428.

Kubota, M., Lin, Y.W., Hamahata, K., Sawada, M., Koishi, S., Hirota, H., and Wakazono, Y. 2000. Cancer chemotherapy and somatic cell mutation. *Mutat. Res.* 470: 93-102.

Kumaravel, T.S., and Jha, A.N. 2006. Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutat Res.* 605(1-2): 7-16.

Kumaravel, T.S., Vilhar, B., Faux, S.P., and Jha, A.N. 2009. Comet Assay measurements: a perspective. *Cell Biol. and Toxicol.* 25: 53-64.

Kyrtopoulos, S.A. 2006. Biomarkers in environmental carcinogenesis research: striving for a new momentum. *Toxicol. Lett.* 162(1): 3-15.

Laantri, N., Jalbout, M., Khyatti, M., Ayoub, W.B., Dahmoul, S., Ayad, M., Bedadra, W., Abdoun, M., Mesli, S., Kandil, M., Hamdi-Cherif, M., Boualga, K., Bouaouina, N., Chouchane, L., Benider, A., Ben-Ayed, F., Goldgar, D., and Corbex, M. 2011. XRCC1 and hOGG1 genes and risk of nasopharyngeal carcinoma in North African countries. *Mol. Carcinog.* 50: 732-737.

Laffon, B., Teixeira, J.P., Silva, S., Roma-Torres, J., Pérez-Cadahía, B., Méndez, J., Pásaro, E., and Mayan, O. 2006. Assessment of occupational genotoxic risk in the production of rubber tyres. *Ann. Occup. Hyg.* 50: 583–592.

Landi, S. 2000. Mammalian class theta GST and difefrential susceptibility to carcinogens: a review. *Mutat. Res.* 463: 247-283.

Lando, C., Hagmar, L., and Bonassi, S. 1998. Biomarkers of cytogenetic damage in human and risk of cancer. The European Study Group on Cytogenetic Biomarkers and Health (ESCH). *Med. Lav.* 89: 124–131.

Lehmann, I., Sack, U., and Lehmann, J. 2011. Metal ions affecting the immune system. In *Metal ions in toxicology: effects, interactions, interdependencies* edited by Sigel, A., Sigel, H. and Sigel, R.K.O. Cambridge, UK: The Royal Society of Chemistry. pp. 157-185.

Lin, D.-X., Tang, Y.-M., Peng, Q., Lu, S.-X., Ambrosone, C.B., and Kadlubar, F.F. 1998. Susceptibility to Esophageal Cancer and Genetic Polymorphisms in Glutathione S Transferases T1 , P1 , and M1 and Cytochrome P450 2E1. *Cancer Epidemiol. Biomarkers Prev.* 7: 1013-1018.

Liu, Q., Cao, J., Liu, Y., Lü, Y.M., Qin, B., Jiang, B., Jiang, L.P., Fu, B.H., Zhao, F.L., Jiang, E.H., Su, X., and Fan, F.Y. 2010. Follow-up study by chromosome aberration analysis

and micronucleus assays in victims accidentally exposed to 60Co radiation. *Health Phys.* 98(6):885-8.

Lotze, F. 1945. Observation respect a la division de los variscides de la Meseta Ibérica. *Publ. Estrag. Geol. Españã* 5:149-166.

Luebke, R.W., Chen, D.H., Dietert, R., Yang, Y., King, M., and Luster, M.I. 2006. The comparative immunotoxicity of five selected compounds following developmental or adult exposure. *J. Toxicol. Environ. Health B* 9: 1–26.

Malanga, M., and Althaus, F.R. 2005. The role of poly(ADP-ribose) in the DNA damage signaling network. *Biochem Cell Biol.* 83(3): 354-364.

Maroni, M. 1983. Recent acquisitions in biological monitoring of human exposure to chloroorganic and organophosphate pesticides. *Proceedings of the International Conference on Environmental Hazards of Agrochemicals in Developing Countries*, 1, pp. 345–371.

Maroni, M., Colosio, C., Ferioli, A., and Fait, A. 2000. Biological Monitoring of Pesticide Exposure: a review. Introduction. *Toxicology* 143: 1-118.

Martínez, V., Creus, A., Venegas, W., Arroyo, A., Beck, J.P., Gebel, T.W., Surrallés, J., and Marcos, R. 2004. Evaluation of micronucleus induction in a Chilean population environmentally exposed to arsenic. *Mutat. Res.* 564(1): 65-74.

Mateuca, R., and Kirsch-Volders, M. 2007. Chromosomal damage. In *State of validation of biomarkers of carcinogen exposure and early effects and their applicability to molecular epidemiology* edited by Farmer P., Kyrtopoulos S. and Emeny J. Poland: Nofer Institute of Occupational Medicine.

Mateuca, R., Lombaert, N., Aka, P.V., Decordier, I., and Kirsch-Volders, M. 2006. Chromosomal changes: induction, detection methods and applicability in human biomonitoring. *Biochimie.* 88(11): 1515-1531.

Mateuca, R.A., Roelants, M., Iarmarcovai, G., Aka, P.V., Godderis, L., Tremp, A., Bonassi, S., Fenech, M., Bergé-Lefranc, J.L., and Kirsch-Volders, M. 2008. hOGG1(326), XRCC1(399) and XRCC3(241) polymorphisms influence micronucleus frequencies in human lymphocytes in vivo. *Mutagenesis* 23(1): 35-41.

McGeer, J., Gerry H., Roman L., Nicholas F., Sappington K., and Drexler J. 2004. *Issue paper on the bioavailability and bioaccumulation of metals Submitted to: US. Environ. Protect. Agency.* Lexington, MA, USA.

McWilliams, R.R., Bamlet, W.R., Cunningham, J.M., Goode, E.L., Andrade, M., Boardman, L.A., and Petersen, G.M. 2008. Polymorphisms in DNA repair genes, smoking, and pancreatic adenocarcinoma risk. *Cancer Res.* 68: 4928–4935.

Mehra, R., and Juneja, M. 2005. Fingernails as biological indices of metal exposure. *J. Biosci.* 30(2): 253-257.

Mertz, W. 1981. The essential trace elements. *Science* 213: 1332-1338.

Milman, N., Mathiassen, B., Hansen, J., and Bohm, J. 1994. Blood levels of lead, cadmium and mercury in a Greenlandic Inuit hunter population from Thule district. *J. Trace Elem. Electrol.* 11: 3-8.

Mo, Z., Gao, Y., Cao, Y., Gao, F., and Jian, L. 2009. An updating meta-analysis of the GSTM1, GSTT1, and GSTP1 polymorphisms and prostate cancer: a HuGE review. *Prostate* 69: 662-688.

Møller, P. 2006. Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA. *Mutat. Res.* 612: 84-104.

Morton, J., Carolan, V. A. and Gardiner, P.H.E. 2002. Removal of exogenously bound elements from human hair by various washing procedures and determination by inductively coupled plasma mass spectrometry. *Anal. Chim. Acta.* 455 (1): 23-34.

Moszczy´nski., P, Zabi´nski, Z, Moszczy´nski, P. Jr., Rutowski, J., Słowi´nski, S., and Tabarowski, Z. 2001. Immunological findings in cigarette smokers. *Toxicol. Lett.* 118: 121–27.

Mudgal V, Madaan N, Mudgal A, Singh R, and Mishra S. 2010. Toxic Metals on Human Health. *The Open Nutraceuticals Journal* 3: 94-99.

Murray, R., Bende,r D., Botham, K., Kennelly, P., Rodwell, V., Weil, P., and Mayes, P. 2009 Micronutrients: Vitamins & Minerals. In *Harper's Illustrated Biochemistry,* Chapter 44 (28th ed.). New York: McGraw-Hill.

Nakata, A., Araki, S., Park, S.-H., Park, J-T., Kim, D.-S., Park, H.-C., and Yokoyama, K. 2006. Decreases in CD8+T, naive (CD4+CD45RA+)T, and B (CD19+) lymphocytes by exposure to manganese fume. *Ind. Health.* 44: 592-597.

National Research Council (NRC) Report. 2006. *Human Biomonitoring for Environmental Chemicals.* The National Academy Press, Washington, DC. pp. 1–213.

Needham, L. L., Calafat, A. M., and Barr, D. B. 2007. Uses and issues of biomonitoring. *Int. J. Hyg. Environ. Health.* 210: 229-238.

Neurauter, G., Schrocksnadel, K., Scholl-Burgi, S., Sperner-Unterweger, B., Schubert, C., Ledochowski, M., and Fuchs, D. 2008. Chronic immune stimulation correlates with reduced phenylalanine turnover. *Curr. Drug Metab.* 9: 622– 627.

Noronha, F., Doria, A., Dubessy, J., and Charoy, B. 1992. Characterization and timing of the different types of fluids present in the barren and ore veins of the W-Sn deposit of Panasqueira, Central Portugal. *Mineral. Deposita* 27: 72-79.

Norppa, H. 1997. Cytogenetic markers of susceptibility: influence of polymorphic carcinogen-metabolizing enzymes. *Environ. Health Perspect.* 105(4): 829-385.

Norppa, H. 2004. Cytogenetic biomarkers and genetic polymorphisms. *Toxicol. Lett.* 149: 309-334.

Nowak, B. and Kozlowski, H. 1998. Heavy metals in human hair and teeth: the correlation with metal concentration in the environment. *Biol. Trace Elem. Res.* 62: 213:228.

OECD. 2008. *Health and the environment.* Policy Briefs are prepared by the Public Affairs Division, Public Affairs and Communications Directorate. They are published under the responsibility of the Secretary-General. Available from: http://www.oecd.org/dataoecd/38/18/40396531.pdf [accessed 16 September 2012].

Oh, E., Lee, E., Im, H., Kang, H.-S., Jung, W.-W., Won, N.H., Kim, E.-M., and Sul, D. 2005. Evaluation of immuno- and reproductive toxicities and association between immunotoxicological and genotoxicological parameters in waste incineration workers. *Toxicology* 210: 65-80.

Olive, P.L., Banath, J.P., and Durand, R.E. 1990a. Detection of etoposide resistance by measuring DNA damage in individual Chinese hamster cells. *J. Natl. Cancer Inst.* 82: 779-783.

Olive, P.L., Banath, J.P., and Durand, R.E. 1990b. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiat. Res.* 122: 86-94.

Orloff, K., Mistry, K., and Metcalf, S. 2009. Biomonitoring for environmental exposures to arsenic. *J. Toxicol. Environ. Health B* 12: 509–524.

Park, J.Y., Park, S.H., Choi, J.E., Lee, S.Y., Jeon, H.S., Cha, S.I., Kim, C.H., Park, J.H., Kam, S., Park, R.W., Kim, I.S., and Jung, T.H. 2002. Polymorphisms of the DNA repair gene xeroderma pigmentosum group A and risk of primary lung cancer. *Cancer Epidemiol. Biomarkers Prev.* 11(10 Pt 1): 993-997.

Parl, F.F. 2005. Glutathione S-transferase genotypes and cancer risk. *Cancer Lett.* 221: 123-129.

Pavanello, S., and Clonfero, E. 2000. Biological indicators of genotoxic risk and metabolic polymorphisms. *Mutat. Res.* 463: 285–308.

Pedersen, E.R., Midttun, Ø., Ueland, P.M., Schartum-Hansen, H., Seifert, R., Igland, J., Nordrehaug, J.E., Ebbing, M., Svingen, G., Bleie, Ø., Berge, R., and Nygård, O. 2011. Systemic markers of interferon-γ-mediated immune activation and long-term prognosis in patients with stable coronary artery disease. *Arterioscler Thromb. Vasc. Biol.* 31: 698-704.

Pemble, S., Schroeder, K.R., Spencer, S.R., Meyer, D.J., Hallier, E., Bolt, H.M., Ketterer, B., and Taylor, J.B. 1994. Human glutathione S-transferase Theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. *Biochem. J.* 300: 271-276.

Peplow, D. and Edmonds, R. 2005. The effects of mine waste contamination at multiple levels of biological organization. *Ecol. Eng.* 24: 101-119.

Quereshi, I. 1982. Trace elements concentration in head hair of the inhabitants of the Rawalpindi-Islamabad area. *J. Radioanal. Chem.* 68: 209-214.

Reis, A.C. 1971. As Minas da Panasqueira. *Bol. de Minas* 8(1): 3-34.

Roberts, M.R., Shields, P.G., Ambrosone, C.B., Nie, J., Marian, C., Krishnan, S.S., Goerlitz, D.S., Modali, R., Seddon, M., Lehman, T., Amend, K.L., Trevisan, M., Edge, S.B., and Freudenheim, J.L. 2011 Single-nucleotide polymorphisms in DNA repair genes and association with breast cancer risk in the web study. *Carcinogenesis* 32: 1223-1230.

Rodrigues Abrantes, J., and Gonzalez, V. 1973 O tratamento mecânico do minério nas Minas da Panasqueira. *Bol. de Minas* 19(4): 239-247.

Rodushkin, I. and Axelsson, M. 2000. Application of double focusing sector field ICP-MS multielemental characterization of human hair and nails. Part II. A study of the inhabitants of northen Sweden. *T. Sci. Tot. Environm.* 262: 21-36.

Roma-Torres, J., Teixeira, J.P., Silva, S., Laffon, B., Cunha, L.M., Méndez, J., and Mayan, O. 2006. Evaluation of genotoxicity in a group of workers from a petroleum refinery aromatics plant. *Mutat. Res.* 604(1-2):19-27.

Rossi, A.M., Hansteen, I.L., Skjelbred, C.F., Ballardin, M., Maggini, V., Murgia, E., Tomei, A., Viarengo, P., Knudsen, L.E., Barale, R., Norppa, H., and Bonassi, S. 2009. Association between frequency of chromosomal aberrations and cancer risk is not influenced by genetic polymorphisms in GSTM1 and GSTT1. *Environ. Health. Perspect.* 117(2): 203-208.

Sachidanandam, R., Weissman, D., Schmidt, S.C., Kakol, J.M., Stein, L.D., Marth, G., Sherry, S., Mullikin, J.C., Mortimore, B.J., Willey, D.L., Hunt, S.E., Cole, C.G., Coggill, P.C., Rice, C.M., Ning, Z., Rogers, J., Bentley, D.R., Kwok, P.Y., Mardis, E.R., Yeh, R.T., Schultz, B., Cook, L., Davenport, R., Dante, M., Fulton, L., Hillier, L., Waterston, R.H., McPherson, J.D., Gilman, B., Schaffner, S., Van Etten, W.J., Reich, D., Higgins, J., Daly, M.J., Blumenstiel, B., Baldwin, J., Stange-Thomann, N., Zody, M.C., Linton, L., Lander, E.S., and Altshuler, D. 2001. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* 409: 928-933.

Salgueiro, A., Ávila, P., Pereira, H., and Silva, E. 2008. Assessment of the Impact of Pollution by Arsenic in the Vicinity of Panasqueira Mine (Portugal). *Proceedings of the geoENV 2008 - 7th International Conference on Geostatistics for Environmental Applications, September 8-10, Southampton, UK.*

Salnikow, K., and Zhitkovich, A. 2008. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chem. Res. Toxicol.* 21(1): 28-44.

Sansoni, P., Cossarizza, A., Brianti, V., Fagnoni, F., Snelli, G., Monti, D., Marcato, A., Passeri, G., Ortolani, C., Forti, E., Fagiolo, U., Passeri, M., and Franceschi, C. 1993. Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. *Blood* 82: 2767–2773.

Sasso, A.F., Isukapalli, S.S., and Georgopoulos, P.G. 2010. A generalized physiologically-based toxicokinetic modeling system for chemical mixtures containing metals. *Theor. Biol. Med. Model.* 7:17.

Schindowski, K., Frohlich, L., Maurer, K., Muller, W.E., and Eckert, A. 2002. Age related impairment of human T lymphocytes' activation: specific differences between CD4(+) andCD8(+) subsets. *Mech. Ageing Dev.* 123: 375–390.

Schrauzer, G., Shrestha, K., and Flores-Arce, M. 1992. Lithium in scalp hair of adults, students, and violent criminals. *Biol. Trace Elem. Res.* 34: 161–176.

Schroecksnadel, K., Wirleitner, B., Winkler, C., and Fuchs, D. 2006. Monitoring tryptophan metabolism in chronic immune activation. *Clin. Chim. Acta* 364: 82-90.

Schulte, P.A., and Perera, F.P. 1993. Validation. *In Molecular Epidemiology.* San Diego, CA: Academic Press. pp. 81–104.

Shahabuddin, S., Al-Ayedi, I., Gad El-Rab, M.O., and Qureshi, M. I. 1998. Age-related changes in blood lymphocyte subsets of Saudi Arabian healthy children. *Clin. Diagn. Lab. Immunol.* 5: 632–635.

Shen, M., Berndt, S.I., Rothman, N., Mumford, J.L., He, X., Yeager, M., Welch, R., Chanock, S., Keohavong, P., Donahue, M., Zheng, T., Caporaso, N., and Lan, Q. 2005. Polymorphisms in the DNA base excision repair genes APEX1 and XRCC1 and lung cancer risk in Xuan Wei, China. *Anticancer Res.* 25(1B): 537-542.

Shinmura, K., Yamaguchi, S., Saitoh, T., Takeuchi-Sasaki, M., Kim, S.R., Nohmi, T., and Yokota, J. 2000. Adenine excisional repair function of MYH protein on the adenine: 8-hydroxyguanine base pair in double-stranded DNA. *Nucleic Acids Res.* 28: 4912–4918.

Singh, N., McCoy, T., Tice, R., and Schneider, L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175: 184-191.

Silva, S.N., Azevedo, A.P., Teixeira, V., Pina, J.E., Rueff, J., and Gaspar, J.F. 2009. The role of GSTA2 polymorphisms and haplotypes in breast cancer susceptibility: a case-control study in the Portuguese population. *Oncol. Rep.* 22(3): 593-598.

Silva, S.N., Tomar, M., Paulo, C., Gomes, B.C., Azevedo, A.P., Teixeira, V., Pina, J.E., Rueff, J., and Gaspar, J.F. 2010. Breast cancer risk and common single nucleotide polymorphisms in homologous recombination DNA repair pathway genes XRCC2, XRCC3, NBS1 and RAD51. *Cancer Epidemiol.* 34(1): 85-92.

Skjelbred, C.F., Svendsen, M., Haugan, V., Eek, A.K., Clausen, K.O., Kure, E.H., Tuimala, J.T., Svendsen, M.V., Norppa, H., and Hansteen, I.L. 2011. Influence of GSTM1, GSTT1, GSTP1, NAT1, NAT2, EPHX1, MTR and MTHFR polymorphism on chromosomal aberration frequencies in human lymphocytes. *Carcinogenesis* 32(3): 399-405.

Slotnick, M., Meliker, J., Avruskin, G., Ghosh, D., and Nriagu, J. 2007. Toenails as a biomarker of inorganic arsenic intake from drinking water and food. *J. Toxicol. Environ. Health A* 70: 148–158.

Smith, M, 2006. Panasqueira the tungsten giant at 100+. Operation Focus. *Int. Mining* 2: 10-14.

Smith, T.R., Levine, E.A., Perrier, N.D., Miller, M.S., Freimanis, R.I., and Lohman, K. 2003. Case LD, Xu J, Mohrenweiser HW, Hu JJ. 2003. DNA-repair genetic polymorphisms and breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.* 12(11 Pt 1): 1200-1204.

Spencer, M.E., Jain, A., Matteini, A., Beamer, B.A., Wang, N.Y., Leng, S.X., Punjabi, N.M., Walston, J.D., and Fedarko, N.S. 2010. Serum levels of the immune activation marker neopterin change with age and gender and are modified by race, BMI, and percentage of body fat. *J. Gerontol. A Biol. Sci. Med. Sci.* 65: 858–865.

Stephens, C. and Ahern, M. 2002. *Worker and Community Health Impacts Related to Mining Operations Internationally. A Rapid Review of the Literature.* Paper prepared for MMSD. London: IIED and WBCSD.

Still, I.H., Vince, P., and Cowell, J.K. 1999. Identification of a novel gene (ADPRTL1) encoding a potential Poly(ADP-ribosyl)transferase protein. *Genomics* 62(3): 533-536.

Strange, R.C., Jones, P.W., and Fryer, A.A. 2000. Glutathione S-transferase: genetics and role in toxicology. *Toxicol. Lett.* 112-113: 357-363.

Sucher, R., Schroecksnadel, K., Weiss, G., Margreiter, R., Fuchs, D., and Brandacher, G. 2010. Neopterin, a prognostic marker in human malignancies. *Cancer Lett.* 287: 13-22.

Sundberg, K., Johansson, A., Stenberg, G., Widersten, M., Seidel, A., Mannervik, B. and Jernstrom, B. 1998. Differences in the catalytic efficiences of allelic variants of glutathione transferase P1-1 towards carcinogenic diol epoxides of polycyclic aromatic hydrocarbons. *Carcinogenesis* 19: 433–436.

Surrallés, J., Falck, G., and Norppa, H. 1996. In vivo cytogenetic damage revealed by FISH analysis of micronuclei in uncultured human T lymphocytes. Cytogenet *Cell Genet.* 75: 151–154.

Suzumura, H., and Arisaka, O. 2010. Cerebro-oculo-facio-skeletal syndrome. *Adv. Exp. Med. Biol.* 685: 210-214.

Takeuchi, T., Hayashi, T., and Takada, J. 1982. Variation of elemental concentration in hair of the Japanese in term of age, sex and hair treatment. *J. Radioanal. Chem.* 70: 29-55.

Taooka, Y., Takeichi, N., Noso, Y., Kawano, N, Apsalikov, K., and Hoshi, M. 2006. Increased T-cell receptor mutation frequency in radiation-exposed residents living near the Semipalatinsk nuclear test site. *J. Radiat. Res.* 47: 179–181.

Taylor, A., Branch, S., Day, M., Patriarca, M., and White, M. 2011. Atomic spectrometry update: clinical and biological materials, foods and beverages. *J. Anal. At. Spectrom.* 26: 653-692.

Taylor, M.W., and Feng, G.S. 1991. Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. *FASEB J.* 5: 2516–2522.

Tell, G., Quadrifoglio, F., Tiribelli, C., and Kelley, M.R. 2009. The many functions of APE1/Ref- 1: not only a DNA repair enzyme. *Antioxid. Redox Sign.* 11: 601-620.

Tetlow, N., and Board, P.G. 2004. Functional polymorphism of human glutathione transferase A2. *Pharmacogenetics* 14(2): 111-116.

Teixeira, J.P., Gaspar, J., Martinho, G., Silva, S., Rodrigues, S., Mayan, O., Martin, E., Farmer, P.B., and Rueff, J. 2004. Aromatic DNA adduct levels in coke oven workers: correlation with polymorphisms in genes GSTP1, GSTM1, GSTT1 and CYP1A1. *Mutat. Res.* 517(1-2): 147-155.

Therman E. 1980. *Human Chromosomes: Structure, Behavior, Effects.* New York: Springer-Verlag. pp 235.

Thygesen, L.C., Hvidtfeldt, U.A., Mikkelsen, S., and Bronnum-Hansen, H. 2011. Qunatification of the healthy worker effect: a nationwide cohort among electricians in Denmark. *BMC Public Health* 11: 571.

Tice, R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., and Sasaki, F. 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* 35: 206-221.

Tulinska, J., Jahnova, E., Dusinska, M., Kuricova, M., Liskova, A., Ilavska, S., Horvathova, M., Wsolova, L., Kyrtopoulos, S., Collins, A., Harrington, V., and Fuortes, L. 2004. Immunomodulatory effects of mineral fibres in occupationally exposed workers. *Mutat. Res.* 553: 111-124.

Tryphonas, H., Fournier, M., Blakley, B., Smits, J., and Brousseau, P. 2005. Structural and functional complexity of the immune system and its relationship to immunotoxicology. In *Investigative Immunotoxicology* edited by Blakley, B.R. and Kouassi, E. Taylor and Francis, Boca Raton. pp. 3-10.

Vahter, M., Akesson, A., Liden, C., Ceccatelli, S., and Berglund, M. 2007. Gender differences in the disposition and toxicity of metals. *Environ. Res.* 104: 85–95.

van Vuuren, A.J., Appeldoorn, E., Odijk, H., Humbert, S., Moncollin, V., Eker, A.P., Jaspers, N.G., Egly, J.M., and Hoeijmakers, J.H. 1995. Partial characterization of the DNA repair protein complex, containing the ERCC1, ERCC4, ERCC11 and XPF correcting activities. *Mutat. Res.* 337(1): 25-39.

Weiss, G., Murr, C., Zoller, H., Haun, M., Winder, B., Ludescher, C., and Fuchs, D., 1999. Modulation of neopterin formation and tryptophan degradation by Th1- and Th2-derived cytokines in human monocytic cells. *Clin. Exp. Immunol.* 116: 435-440.

Werck-Reichhart. D, and Feyereisen, R. 2000. Cytochromes P450: a success story. *Genome Biol.* 1(6): REVIEWS3003.

Wick, G., Romen, M., Amberger, A., Metzler, B., Mayr, M., Falkensammer, G., and Xu, Q. 1997. Atherosclerosis, autoimmunity, and vascular-associated lymphoid tissue. *FASEB J.* 11: 1199–1207.

Widner, B., Werner, E. R., Schennach, H., Wachter, H., and Fuchs, D. 1997. Simultaneous measurement of serum tryptophan and kynurenine by HPLC. *Clin. Chem.* 43: 2424–2426.

Wilson, D.M., Kim, D., Berquist, B.R., and Sigurdson, A.J. 2011. Variation in base excision repair capacity. *Mutat. Res.* 711: 100-112.

World Health Organization (WHO). 1995. *IPCS Environmental Health Criteria 165 Inorganic Lead.*

World Health Organization (WHO). 2001. *Biomarkers in risk assessment: validity and validation. Geneva. EHC 222.*

Xanthoudakis, S., Miao, G.G., and Curran, T. 1994. The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. *Proc. Natl. Acad. Sci. USA* 91: 23-27.

Yamane, A., Kohno, T., Ito, K., Sunaga, N., Aoki, K., Yoshimura, K., Murakami, H., Nojima, Y., and Yokota, J. 2004. Differential ability of polymorphic OGG1 proteins to suppress mutagenesis induced by 8-hydroxyguanine in human cell in vivo. *Carcinogenesis* 25(9):1689-1694.

Yu, H., Zhao, H., Wang, L.E., Liu, Z., Li, D., and Wei, Q. 2012. Correlation between base-excision repair gene polymorphisms and levels of in-vitro BPDE-induced DNA adducts in cultured peripheral blood lymphocytes. *PLoS One* 7(7): e40131.

Yuan, H., He, S., He, M., Niu, Q., Wang, L., and Wang, S. 2006. A comprehensive study on neurobehavior, neurotransmitters and lymphocyte subsets alteration of Chinese manganese welding workers. *Life Sci.* 78: 1324-1328.

Zienolddiny, S., Campa, D., Lind, H., Ryberg, D., Skaug, V., Stangeland, L., Phillips, D.H., Canzian, F., and Haugen, A. 2006. Polymorphisms of DNA repair genes and risk of non-small cell lung cancer. *Carcinogenesis* 27(3): 560-567.

Zijno, A., Verdina, A., Galati, R., Leopardi, P., F. Marcona, Andreoli, C., Rossi, S. and Crebelli, R. 2006. Influence of DNA repair polymorphisms on biomarkers of genotoxic damage in peripheral lymphocytes of healthy subjects. *Mutat. Res.* 600: 184-192.

VIII. ANNEXES

ANNEX I

PROTOCOLO DE COOPERAÇÃO ENTRE A
ADMINISTRAÇÃO REGIONAL DE SAÚDE DO CENTRO, IP
E O INSTITUTO NACIONAL DE SAÚDE DR. RICARDO JORGE, IP

O desenvolvimento técnico-científico actual permite a justa procura de modelos explicativos plurietiológicos dos fenómenos de saúde e de doença das populações humanas.

O conhecimento destes modelos, tendencialmente complexos, exige o fortalecimento da capacidade de investigação através de estratégias de cooperação multidisciplinar, intersectorial e interinstitucional.

A exposição a factores ambientais, naturais ou resultantes da actividade humana, bem como determinados comportamentos, podem contribuir, determinar ou agir como factores condicionadores de diversas patologias. Recentemente têm sido múltiplas as situações em que é imputado um acréscimo de morbilidade por comportamentos ou exposição a factores ambientais.

A Administração Regional de Saúde do Centro (ARSC) através do seu Departamento de Saúde Pública e Planeamento (DSPP) têm consignado no seus estatutos, nomeadamente intervenções no domínio da caracterização e monitorização do estado de saúde da população, vigilância epidemiológica dos fenómenos de saúde e dos seus determinantes e promoção da investigação em saúde (Portaria N.º 650/2007 de 30 de Maio).

Neste contexto, a ARSC, pretende estabelecer o presente protocolo de cooperação com o Instituto Nacional de Saúde Dr. Ricardo Jorge, IP (INSA), facilitador de projectos de colaboração, nomeadamente na vertente laboratorial.

Administração Regional de Saúde do Centro IP, adiante designada por ARSC, representada pelo Presidente do seu Conselho Directivo, Dr. João Pedro Travassos Pimentel e o Instituto Nacional de Saúde Dr. Ricardo Jorge, IP (INSA), representado pelo Presidente do seu Conselho Directivo, Prof. Doutor José Pereira Miguel, estabelecem entre si o presente protocolo de cooperação, que se rege pelas cláusulas seguintes:

Cláusula 1ª (objectivos)

A cooperação entre a ARSC e o INSA IP desenvolver-se-á em domínios técnicos e científicos de interesse comum, designadamente no âmbito da Saúde Pública e Ambiental da Região Centro do País.

Cláusula 2ª (formas de cooperação)

A cooperação entre a ARSC e o INSA IP revestirá as formas que, em cada momento e por mútuo acordo, forem tidas por mais adequadas, designadamente as seguintes:

a) Acesso a informação bibliográfica, técnica e científica;
b) Realização de projectos de investigação;
c) Elaboração de estudos técnico-científicos de âmbito regional ou local;
d) Formação;
e) Consultadoria;
f) Prestação de Serviços.

Cláusula 3ª (prestação de serviços)

A prestação de serviços (incluído o pagamento que venha a implicar) rege-se por regras estabelecidas caso a caso e por mútuo acordo, salvaguardadas que sejam as normas legais e regulamentos aplicáveis.

Cláusula 4ª (publicações)

a) A eventual publicação de estudos efectuados no âmbito das alíneas b) e c) da cláusula 2ª carece de autorização da ARSC e do INSA IP.
b) As publicações a que se refere a alínea anterior apenas responsabilizam os seus autores.
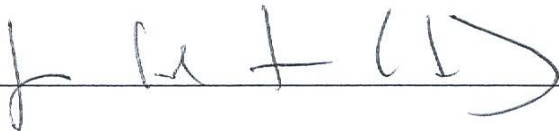
Cláusula 5ª (duração)

O protocolo entrará em vigor na data da sua assinatura e terá a duração de dois anos.

Findo o primeiro período de duração, considera-se automaticamente renovado por sucessivos períodos de igual duração se não for denunciado por qualquer das partes.
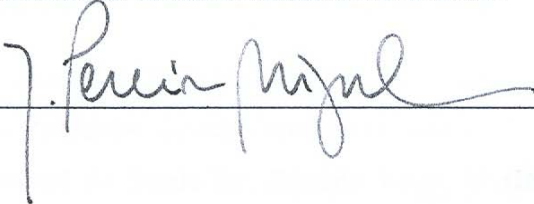
Cláusula 6ª (revisão, suspensão ou termo)

O protocolo pode ser revisto a qualquer tempo por mútuo acordo das partes. Qualquer destas pode declarar a sua suspensão ou o seu termo desde que avisada a outra com antecedência mínima de 90 dias.

Coimbra, 16 de Jane de 2008

O Presidente do Conselho Directivo da ARSC

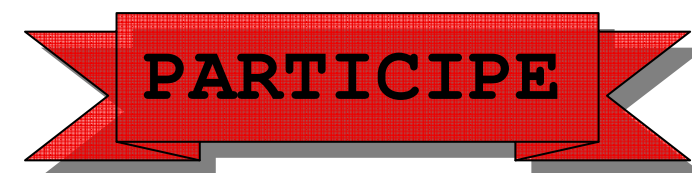O Presidente do concelho directivo do INSA IP

ANNEX II

Este estudo faz parte de um projecto de doutoramento que está a ser desenvolvido no Departamento de Saúde Ambiental do Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P.

Em caso de dúvida não hesite em contactar:

Dra. Patrícia Coelho

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Instituto_Nacional de Saúde
Doutor Ricardo Jorge

Colaboração

FCS Faculdade de Ciências da Saúde

ARSC ADMINISTRAÇÃO REGIONAL DE SAÚDE DO CENTRO

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Instituto Nacional de Saúde
Doutor Ricardo Jorge

Estudo dos efeitos na saúde das populações da contaminação ambiental resultante das actividades mineiras

PARTICIPE

INFORMAÇÕES RELEVANTES SOBRE O ESTUDO

Importância

A saúde é condição indispensável para o bem estar do ser humano, e, como tal, justifica todos os esforços dispendidos na sua manutenção.

O nível de saúde de uma comunidade é influenciado pelo meio ambiente podendo este causar o aparecimento de problemas de saúde que afectem os seus elementos.

É do conhecimento geral que as actividades mineiras têm um impacte ambiental significativo com consequentes efeitos negativos na saúde humana.

É então importante estudar estes efeitos de modo a que estes possam ser minorados.

Objectivo

Este projecto pretende avaliar até que ponto a saúde das populações residentes na envolvente das Minas da Panasqueira é afectada pelos resíduos por estas gerados, nomeadamente pela presença de metais pesados.

Locais de estudo

Neste projecto serão estudadas populações situadas na envolvente das Minas da Panasqueira (populações expostas) e uma população controlo.

Participação

Pede-se a colaboração de cerca de 75 pessoas de cada povoação, com idade superior a 18 anos e que residam permanentemente na localidade há mais de 5 anos.

As populações serão informadas atempadamente da data e local de encontro. Agradecíamos que comparecesse e que:

⇒ respondesse a um questionário de saúde;
⇒ permitisse a colheita de amostras de sangue e de uma pequena madeixa de cabelo;
⇒ que nos fornecesse uma amostra de urina (1 frasco) e algumas unhas das mãos e pés (cortadas normalmente).

As conclusões do estudo serão tornadas públicas.

Importância dos resultados

Os resultados obtidos permitirão:
⇒ uma melhor caracterização dos riscos para a saúde pública da área em estudo o que possibilitará a adopção de cuidados de saúde adequados à situação em causa;
⇒ realização de obras de requalificação melhorando a qualidade de vida dos seus residentes;
⇒ implementação de programas de vigilância mais adequados.

A publicação dos resultados do estudo nunca incluirá o nome dos participantes ou qualquer elemento que permita identificá-los.

Quando se fala em saúde e bem estar, a ajuda de todos é fundamental.

Com a certeza que vai participar neste estudo,

MUITO OBRIGADO!

ANNEX III

Ministério da Saúde

INSTITUTO NACIONAL DE SAÚDE
Dr. Ricardo Jorge

"ESTUDO DOS EFEITOS NA SAÚDE DAS POPULAÇÕES DA CONTAMINAÇÃO AMBIENTAL RESULTANTE DAS ACTIVIDADES MINEIRAS"

Declaração de Consentimento

Fui informado sobre os objectivos do estudo e compreendi com clareza o que me é pedido, como participante.

Fui ainda informado que:

1- Os dados que fornecer, bem como os resultados das análises que forem efectuadas sobre as amostras do meu sangue, urina, unhas e cabelo serão estritamente confidenciais. Assim que os procedimentos do estudo o permitam esses dados e resultados serão tornados anónimos, isto é, deixarão de poder ser relacionados com a minha identificação;
2- Todos os investigadores e técnicos que utilizem esses dados estarão obrigados a segredo profissional;
3- Os resultados do estudo que venham a ser tornados públicos nunca incluirão o meu nome ou qualquer elemento que permita identificar-me.

Nestas condições, declaro que aceito participar no estudo, disponibilizando-me para:

1- Ser entrevistado e prestar informações sobre vários aspectos respeitantes a doenças de que tenha sofrido bem como a algumas características pessoais relevantes;
2- Permitir a colheita de uma amostra de sangue, urina, cabelo e unhas para realizar várias análises relacionadas com este estudo ou com outros que sejam realizados no futuro.

Data: _____/_____/_____

Nome do participante:_____

Assinatura:_____

ANNEX IV

"ESTUDO DOS EFEITOS NA SAÚDE DAS POPULAÇÕES DA CONTAMINAÇÃO AMBIENTAL RESULTANTE DAS ACTIVIDADES MINEIRAS"

Questionário de selecção

(critérios de exclusão)

1 – Tem idade inferior a 18 anos? Sim ❐   Não ❐

    Se sim, terminar a entrevista.

2 – Reside nesta localidade continuamente há menos de 5 anos? Sim ❐   Não ❐

    Se sim, terminar a entrevista.

ANNEX V

QUESTIONÁRIO PARA AVALIAÇÃO DOS EFEITOS NA SAÚDE DAS POPULAÇÕES DA CONTAMINAÇÃO AMBIENTAL RESULTANTE DAS ACTIVIDADES MINEIRAS

Código de identificação: _____   **Data:** _____/_____/_____

Caso ❏   **Controlo** ❏

1 - Nome: _____

2 - Residência: _____
_____

3 – Sexo: Feminino ❏   Masculino ❏

4 - Data de Nascimento: _____/_____/_____

5 - Estado Civil:

Solteiro(a) ❏   Casado(a) ❏   Divorciado(a) ❏   Viuvo(a) ❏
Em união de facto ❏

6 - Habilitações literárias:

Não sabe ler/escrever ❏   Ensino Secundário ❏

Sabe ler/escrever sem concluir o 1º ciclo ❏   Ensino Superior Politécnico ❏

1º Ciclo do Ensino Básico ❏   Ensino Superior Universitário ❏

2º Ciclo do Ensino Básico ❏   Mestrado ❏

3º Ciclo do Ensino Básico ❏   Doutoramento ❏

7 – Profissão/Ocupação/Função: _____

_____

7.1 – Já trabalhou em alguma mina? Sim ☐   Não ☐

Se sim: qual? _____

qual era a sua função? _____

durante quanto tempo trabalhou? _____ anos

há quanto tempo? _____

7.2 – Profissões anteriores

Profissão/Função	Anos
_____	_____
_____	_____
_____	_____

8 – Hábitos tabágicos

8.1 – É actualmente fumador? Sim ☐   Não ☐

Se sim: com que idade começou a fumar? _____ anos

quantos cigarros fuma por dia? _____

8.2 – Alguma vez fumou? Sim ☐   Não ☐

Se sim: com que idade começou a fumar? _____ anos

quantos cigarros fumava por dia? _____

com que idade deixou de fumar? _____ anos

deixou de fumar porque _____

_____

9 – Consumo de álcool

9.1 – Consome: Sim ☐   Não ☐

Se sim :que quantidade consome em média por dia? _____

que tipo de bebida consome habitualmente? _____

_____

10 – Faz vigilância da sua saúde? Sim ❒   Não ❒

   10.1 – O que o leva a recorrer aos serviços de saúde?

      Doença ❒   Vigilância ❒   Ambas ❒

11 – Alguma vez lhe disseram que tinha bronquite crónica? Sim ❒   Não ❒

      Se sim: tomou medicação? Sim ❒   Não ❒

12 – Costuma ter pieira?

	Sim	Não
A – Em ambiente com fumo	❒	❒
B – Em ambiente empoeirado	❒	❒
C – Só quando está constipado(a)	❒	❒

Constipa-se quantas vezes por ano? _____

13 – É habitual ter:

	Sim	Não	Quantas vezes/ano
A – Nariz entupido	❒	❒	_____
B – Pingo no nariz	❒	❒	_____
C – Espirros	❒	❒	_____
D – Rouquidão	❒	❒	_____
E – Dificuldade em respirar	❒	❒	_____

14 – Costuma ter tosse com expectoração? Sim ❒   Não ❒

      Se sim: só raramente   ❒

             só de manhã   ❒

             durante a maior parte do dia   ❒

             muitas vezes no ano   ❒

15 – Tem filhos: Sim ☐　　　　　Não ☐

Se sim: quantos? _____

Se nunca teve filhos isso aconteceu porque:

A - Nunca quis ☐

B - Tem esterilidade ☐

C - Outra razão_____

_____

Se é homem passar para a questão 16.

15.1 - Teve problemas em engravidar? Sim ☐　　　　　Não ☐

Se sim: qual a razão? _____

15.2 – Teve algum aborto? Sim ☐　　　　　Não ☐

16 – Notou, em algum momento, alterações na cor e/ou textura da pele?

Se sim: quais? _____

_____

15.1 – A nível bucal, notou alterações de cor (dentes, gengivas,..)? _____
_____

17 – Nota alterações no cheiro? Sim ☐　　　　　Não ☐

Se sim: qual? _____

18 – Habitualmente come com apetite? Sim ☐　　　　　Não ☐

18.1 – Nota alterações no seu paladar? Sim ☐　　　　　Não ☐

Se sim: qual? _____

19 – O seu peso tem tido grandes oscilações? Sim ☐　　　　　Não ☐

20 – Considera o funcionamento dos seus intestinos normal? Sim ❐   Não ❐

Se não: diarreia   ❐

obstipação   ❐

21 – Quando há vento é habitual sentir irritação nos olhos (olhos vermelhos)?

Sim ❐   Não ❐

22 – Nos dois últimos anos que doenças teve?

_____

_____

_____

(Especial atenção aos problemas renais, hepáticos e osteológicos(principalmente fracturas)!!!

Atenção também a doenças como Psoriase, Artrite Reumatóide, Doença de Crown e

(Re)Colite Ulcerativa)

22.1 – Toma habitualmente medicamentos receitados pelo seu médico?

Sim ❐   Não❐

Se sim: quais? _____

23 – No último ano fez algum tipo de exame radiológico (ex: Raios X)? Sim ❐   Não ❐

Se sim: qual? _____

24 – Nos últimos anos foi submetido(a) a alguma intervenção cirúrgica? Sim ❐   Não ❐

Se sim: qual? _____

25 – Tomou alguma vacina nos últimos 12 meses? Sim ❐   Não ❐

Se sim: qual? _____

26 – Nos últimos anos faleceu algum familiar directo? Sim ❏   Não ❏

        Se sim: qual foi a causa? _____

27 – Abastecimento de água:

Rede pública dentro de casa   ❏

Rede pública fora de casa   ❏

Outro, qual? _____

   27.1 – Se é fora de casa faz tratamento à água que bebe? Sim ❏ Não ❏

        Se sim: qual? _____

28 – Dedica algum do seu tempo á produção agrícola? Sim ❏   Não ❏

        Se sim: consome esses produtos? _____

        qual a proveniência da água com que rega esses produtos? _____

        _____

        nos últimos 3 anos usou pesticidas agrícolas? Sim ❏   Não ❏

Está interessado em participar na próxima fase do estudo na qual será colhida uma amostra de sangue, urina, alguns fios de cabelo e uma amostra de unhas?

Sim ❏   Não ❏

Contacto: _____

ANNEX VI

"ESTUDO DOS EFEITOS NA SAÚDE DAS POPULAÇÕES DA CONTAMINAÇÃO AMBIENTAL RESULTANTE DAS ACTIVIDADES MINEIRAS "

COLHEITA DE AMOSTRAS BIOLÓGICAS – SANGUE, URINA, CABELOS E UNHAS

Código de identificação: _____

Amostras fornecidas/colhidas:

Sangue ☐   **Data:** ____ / ____ / _____   **Hora:** _____ h _____ m

Urina ☐   **Data:** ____ / ____ / _____   **Hora:** _____ h _____ m

Fios de cabelo ☐   **Data:** ____ / ____ / _____   **Hora:** _____ h _____ m

Unhas ☐   **Data:** ____ / ____ / _____   **Hora:** _____ h _____ m

Cabelo pintado?   Sim ☐   Não ☐

Unhas pintadas?   Sim ☐   Não ☐

Amostras colhidas por:

Sangue: _____

Fios de cabelo: _____

Observações:_____
_____
_____
_____
_____

QUESTIONÁRIO COMPLEMENTAR

1. Quanto tempo despende em média na produção agrícola?

    1.1. Por dia: _____ horas.

    1.2. Por semana: _____ dias.

2. Consome produtos agrícolas cultivados aqui na aldeia? Sim ❐   Não ❐

3. Quanto à dieta, come mais:

    vegetais ❐

    carne ❐

    peixe ❐

    mistura ❐

4. Come peixe muitas vezes por semana?   Sim ❐   Não ❐

    4.1. Quantas? _____

5. Come marisco muitas vezes por semana?   Sim ❐   Não ❐

    5.1. Quantas? _____

    5.2. Ultima vez que comeu: ____ / ____ / _____

6. Bebe muito chá? Sim ❐   Não ❐

    6.1. Quantas vezes por semana? _____

    6.2. Quantas chicaras? _____

7. Etnicidade:

    Caucasiano ❐

    Negro ❐

    Asiático ❐

    uma mistura ❐

PUBLICAÇÕES DE SUPORTE À TESE DE DOUTORAMENTO

Geno- and Immunotoxic Effects on Populations Living Near a Mine: A Case Study of Panasqueira Mine in Portugal

Patrícia Clara dos Santos Coelho [a c] , Julia García-Lestón [b] , Susana Pinho e Silva [a] , Carla Sofia Trindade da Costa [a c] , Solange Cristina Bastos da Costa [a c] , Marta Isabel Correia Coelho [a] , Blanca Laffon Lage [b] , Eduardo Pásaro Mendez [b] & João Paulo Fernandes Teixeira [a] [c]

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GENO- AND IMMUNOTOXIC EFFECTS ON POPULATIONS LIVING NEAR A MINE: A CASE STUDY OF PANASQUEIRA MINE IN PORTUGAL

Patrícia Clara dos Santos Coelho[1,3], Julia García-Lestón[2], Susana Pinho e Silva[1], Carla Sofia Trindade da Costa[1,3], Solange Cristina Bastos da Costa[1,3], Marta Isabel Correia Coelho[1], Blanca Laffon Lage[2], Eduardo Pásaro Mendez[2], João Paulo Fernandes Teixeira[1,3]

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Mining industry is a vital economic sector for many countries but it is also one of the most hazardous activities, both occupationally and environmentally. Existing studies point to several adverse effects on communities' health living near mines, effects such as mesothelioma and respiratory illnesses. Results achieved in a geochemical sampling campaign undertaken in the vicinity of São Francisco de Assis village showed an anomalous distribution of some heavy metals in soils and waters. To evaluate the effects of mining activities on human health produced by these conditions, a group of 28 individuals from São Francisco de Assis village was examined for some biological endpoints. A nonexposed group (30 individuals) with the same demographic characteristics without exposure to genotoxic compounds was also studied and data obtained from both groups compared. Results of the T-cell receptor mutation assay and micronucleus (MN) test showed significant increases in the frequencies of both mutations and MN in exposed subjects compared to controls. Data obtained in the analysis of the different lymphocyte subsets demonstrated significant decreases in percentages of CD3+ and CD4+ cells, and a significant increase in percentage of CD16/56+ cells, in exposed individuals. The results of the present study indicate an elevated risk of human environmental contamination resulting from mining activities, emphasizing the need to implement preventive measures, remediation, and rehabilitation plans. This would lead to a reduction in cancer risk not only for this particular population but for all populations exposed under similar conditions.

Mining is one of the oldest activities in human civilization, a vital economic sector for many countries; however, mining is also one of the most hazardous activities in environmental and public health context. During these processes several toxic wastes are produced and released into the surrounding environment, resulting in pollution of air, drinking water, rivers, and soils (Coelho et al. 2007). Mining activities produce several health impacts in miners and communities living near the mine site that may persist even when the mine is abandoned (Heyworth 1990; U.S. EPA 1992; Ladou 1995).

Environmental impact assessment is a crucial tool for maintaining and improving environmental quality and related human health, along with economic development (Health Canada 2004). The impact of mining activities on the community health occurs at various lev-

els, namely, adverse health effects that result from environmental exposure to contaminated air, water, and soil pollution. Existing studies on populations living near mine sites point to several adverse effects on communities' health, such as mesothelioma, and respiratory illnesses (Stephens and Ahern 2001). It is worth noting that many adverse effects are only noted in susceptible groups, such as children and elder people, since their health is more likely to be affected (Stephens and Ahern 2001). Moreover, chronic effects are an important issue that needs to be taken into account.

From the results achieved from a geochemical sampling campaign undertaken in the vicinity of São Francisco de Assis village (a small village near Panasqueira Mine), Salgueiro et al. (2008) concluded that a significant geochemical dispersion, with anomalous patterns, occurs downstream mine tailings, in soils (Avila et al. 2008). Data demonstrated the anomalous distribution of some heavy metals and also arsenic in soils. Furthermore, when comparing the mean values of Casinhas (small river flowing through São Francisco de Assis village) and Zêzere River with the mean values of the background stream sediments, it was apparent there was a moderate to strong enrichment of arsenic (As) ($157\times$; $269\times$), cadmium (Cd) ($34\times$; $59\times$), copper (Cu) ($27\times$; $5\times$) and zinc (Zn) ($7\times$; $2\times$) (Ávila et al. 2008) with values presented in the following ratios: (Casinhas/Backgroud; Zêzere River /Background).

Certain metals are known to produce mutations, and were demonstrated to produce cancer in a variety of animal species (Bernstam and Nriagu 2000; Yang et al. 2008). Based on epidemiological data some of these metals were classified as human carcinogens, namely, As and Cd (Reid et al. 1993; Yang et al. 2008). These two metals were classified as known human carcinogens by the International Agency for Research on Cancer (IARC) and ranked first and seventh, respectively, on a list of top 20 hazard substances by the Agency for Toxic Substances and Disease Registry (ATSDR). Therefore, it is essential to determine environmental and occupational exposure to these substances.

Arsenic is a nonmetal or metalloid that in its pure form has a metallic-gray color, with no odor or taste, so its presence in food, water, and air remains unnoticed. In certain areas As concentration increases considerably, depending on the geochemical characteristics of the area or human activities, such as mining, industrial processes that use carbon as an energy source, glass manufacturing industry, and agriculture (Léonard and Lauwerys 1980; Bernstam and Nriagu 2000). The major route of human exposure to As is through metal dissolved in water (Tsai et al. 1998), but exposure also occurs through contact with contaminated soils, inhalation of particles, or ingestion of contaminated food. In the body As is biotransformed through several reactions, including oxidation/reduction and methylation reactions.

The health risks related to exposure to As are associated with chemical form, concentration, and exposure time. The effects of chronic exposure to As may delay 5 to 20 yr to appear themselves (WHO 2004). Individuals exposed to As exhibit an increased incidence of various types of cancer (Tsai et al. 1998; Steinmaus et al. 2007; Orloff et al. 2009), which is the basis for the International Agency for Research on Cancer (IARC) classification of As and its compounds as carcinogenic to humans (IARC 2004). Several studies in vitro and in vivo showed that As is genotoxic and capable of inducing cytogenetic changes such as micronuclei (MN) (Vega et al. 1995; Basu et al. 2002; Dopp et al. 2004) and DNA damage, including single- and double-strand breaks or alkali-labile sites detected by alkaline comet assay (Basu et al. 2005; Palus et al. 2005; Mouron et al. 2006). It was shown in several studies that As is able to modulate the immune system at environmentally relevant exposure levels (Vega et al. 1999; Meng and Meng 2000). Arsenic may produce inhibition or induction of proliferative responses in animals and humans depending on the dose. At low doses As induced proliferation of lymphocytes (Vega et al. 1999; Meng and Meng 2000), while high doses inhibited proliferative

response (Gonsebatt et al. 1994; Meng and Meng 2000). Changes in lymphocyte subsets indicative of changes in activation processes of T cells were also observed (Soto-Peña et al. 2006). Although there is some information from different animal models, studies on the immunotoxic effects of human exposure to As are scarce.

Cadmium (Cd) is a silver-white, lustrous, malleable and flexible metal that occurs naturally in the earth crust at low concentrations. Exposure to Cd occurs primarily through ingestion of contaminated food or water or through inhalation of tobacco smoke or polluted air. After exposure, this element accumulates in the body, mainly in kidneys and liver. Cadmium exerts adverse effects on humans with a biological half-life of 20 to 30 yr (Goering et al. 1995). After chronic exposure to Cd adverse effects may occur in the renal, cardiovascular, hepatic, respiratory, and reproductive systems (IARC 1993).

Several in vitro studies indicated that Cd and its compounds possessed mutagenic potential, being able to induce damage in chromosomes, such as chromosomal aberrations (CA), and also produced an increase in DNA damage detected by comet assay (DEFRA and EA 2002). The effects of Cd on the immune responses vary based on route of exposure, dose, and endpoint determined. Immunomodulation effects induced by this element include quantitative effects and functional lines in phagocytic and natural killer cells, and antibody response to various antigens. The susceptibility of the immune system to exposure to Pb and Cd is well known (Karakaya et al. 1994; Yucesoy et al. 1997; Luebke et al. 2006). Jung et al. (2003) also demonstrated the ability of Cd, Co, and Cr(VI) to suppress proliferation of B and T cells in animal or in vitro experiments. Nevertheless, although the studies are numerous, the observed effects are divergent.

The main objective of this study was to determine the adverse health effects on a population living near a mine environmentally exposed to metals, predominantly As and Cd, known to induce genotoxicity and immuntoxicity by using a multistage approach in order to integrate different biomarkers.

METHODS

Study Population

The study population consisted of 28 individuals living in São Francisco de Assis village (11 men and 17 women), and 30 individuals nonenvironmentally or occupationally exposed to mining activities, working mainly in administrative offices matched by age, gender, lifestyle, and smoking habits. The choice of Panasqueira Mine (Sn-W mineralization) in central Portugal (40°9'57" N, 7°45'37" W) as object of study for this project was due to its past and current activity, which gave rise to large tailings and dams. In addition, the existence of small villages around the mine site, such as São Francisco de Assis village (40°8'33.78" N, 7°44'9.02" W), with a population strongly dependent on land use and water for agriculture, drinking, and cattle breeding, is an important factor driving such choice. Another aspect of major importance is the fact that the main river flowing in the area (Zêzere river) feeds the Castelo do Bode dam (located 90 km downstream the mine), the principal water supply of Lisbon. Panasqueira mine began its mining activity in 1896 and the tailings disposal occurred over a period reported to be approximately 90 yr. It is important to note that in the last years an increase in production was observed. The existence of a huge tailing and two mud dams around São Francisco de Assis village needs to be emphasized. Tailings and river dams exposed to atmospheric conditions for long periods tend to be affected in their chemical, mineralogical, physical, and geotechnical conditions. Climate, which can be an important instability factor, is aggressive in the mining area, with hot and dry periods during summer and very cool, rainy and windy periods during winter. Rainwater runoff and percolation are responsible for the leaching of the tailing materials and formation of acid drainage (Salgueiro et al. 2008).

TABLE 1. Characteristics of the Study Population

	Exposed group	Control group
Number of subjects	28	30
Gender	17 females	20 females
	11 males	10 males
Age (yr)[a]	59 ± 13 (22–71)	54 ± 6 (46–68)

[a]Mean ± SD.

Selection criteria were living in the villages for at least 5 yr and age over 18 yr. The characteristics of both groups are described in Table 1. Health conditions, medical history, medication, diagnostic tests (x-rays etc.), and lifestyle factors were assessed by means of questionnaires. Subjects also provided information with regard to specific symptoms related to As and Cd exposure and chronic respiratory diseases such as bronchitis and others, water supply, agriculture practice, including the usage of pesticides, and diet. All subjects were fully informed about the procedures and objectives of this study, and each subject signed an informed consent form prior to the study. Ethical approval for this study was obtained from the Institutional Ethical Board of the Portuguese National Institute of Health.

Sample Collection

Blood samples were collected by venipuncture in heparinized sterile tubes for genotoxicity tests, and in sterile tubes containing ethylenediamine tetraacetic acid (EDTA) for immunotoxicity assays. Samples were transported under refrigeration and kept at +4°C until analyses. All samples were coded and analyzed under blind conditions.

Micronucleus (MN) Test

Aliquots of 0.5 ml heparinized whole blood were used to establish duplicate lymphocyte cultures for cytokinesis-blocked MN test, as described in Costa et al. (2006). To determine the total number of MN in binucleated cells, in total 1,000 binucleated cells with well-preserved cytoplasm (500 per replicate) were scored for each subject. MN were scored blindly by the same reader and identified

according to the criteria defined by Fenech (2007) using 500× magnification.

T-Cell Receptor (TCR) Mutation Assay

Isolation of mononuclear leukocytes was performed using BD Vacutainer CPT cell preparation tubes with sodium heparin (Becton Dickinson), following the manufacturer's instructions. TCR mutation assay was carried out by a flow cytometric methodology. Cell suspensions were prepared following the protocol described by García-Lestón et al. (2011), and subsequently analyzed by a FACScalibur flow cytometer with Cell Quest Pro software (Becton Dickinson). A minimum of 2.5×10^5 lymphocyte-gated events were acquired, and mutation frequencies of TCR (TCR-Mf) were calculated as the number of events in the mutant cell window (CD3–CD4$^+$ cells) divided by the total number of events corresponding to CD4$^+$ cells.

Immunotoxicity Assay

The percentages of different lymphocyte subsets, namely, T lymphocytes (CD3$^+$ lymphocytes), T helper (Th) lymphocytes (CD4$^+$ lymphocytes), T cytotoxic (Tc) lymphocytes (CD8$^+$ lymphocytes), B lymphocytes (CD19$^+$ lymphocytes), and natural killer (NK) cells (CD16$^+$ and CD56$^+$ lymphocytes), were evaluated by flow cytometry as described in García-Lestón et al. (2010). Analysis was performed on a FACScalibur flow cytometer using Cell Quest Pro software (Becton Dickinson). At least 10^4 events in the lymphocytes window were acquired.

Statistical Analysis

Student's t-test was applied to assess statistical differences between means. Fisher's exact test for homogeneity or independence was applied to assess the relationship between categorical variables. Multifactorial analysis was performed to evaluate the contribution of potential confounding factors (age and gender) to the response variables considered. Pearson's correlation analysis was used for the estimation

of correlations between parameters. The level of significance was set at $p < .05$. All statistical analyses were performed with the software SPSS 17.0 for Windows statistical package.

RESULTS

The general characteristics of the studied populations are summarized in Table 1. In total, 28 exposed individuals (11 men and 17 women) and 30 controls (10 men and 20 women) were involved in this study. There were no significant differences in age or gender distribution between the two groups. All individuals analyzed were nonsmokers. Analysis of genotoxicity biomarkers presented in Table 2 showed significant differences between populations in TCR-Mf and MN rate, with higher values in exposed compared to controls. A positive and significant correlation between these two variables was also obtained ($r = .295$).

Figure 1 shows the different lymphocyte subsets analyzed. Exposed individuals presented significantly lower %CD3$^+$ and %CD4$^+$ cells accompanied by significantly higher %CD16/56$^+$ cells than controls. The rest of the

immunotoxicity parameters assessed, including %CD8$^+$ lymphocytes, %CD4$^+$/%CD8$^+$ ratio, and %CD19$^+$ cells, were not significantly affected by exposure.

Associations between genotoxicity variables analyzed and lifestyle factors, namely, gender and age, were also evaluated (Table 3). No significant effect of any of the two factors was observed on the TCR-Mf. With respect to MN frequency, a significant effect of gender was obtained with increased frequency in women. In addition, significant correlations were achieved between age and the two genotoxicity biomarkers evaluated ($r = .298$ for TCR-Mf and $r = .265$ MN frequency).

DISCUSSION

Somatic genetic changes are important in cancer development because almost all cancer cells show genomic instability (Balmain et al. 2003). This instability may be induced by inherited mutations in genes, or mutations that are acquired in somatic cells during tumor development (Balmain et al. 2003). The TCR mutation assay is a simple and quick method for evaluating the mutagenic effects in human populations, requiring only a small volume of peripheral blood. The TCR mutation assay was shown to be a sensitive indicator for exposure to several polluting agents including radiation and chemicals (Kubota et al. 2000; Taooka et al. 2006), and determines the genotoxicity associated with exposures experienced in the period of time ranging from several months to 2–3 yr (Taooka et al. 2006). Therefore, the significant elevation in TCR-Mf found in this study in the exposed population indicates mutagenic

TABLE 2. MN and TCR Mutation Frequencies in Control and Exposed Groups, as Mean ± SE (Range)

	n	MN ‰	n	TCR-Mf $\times 10^{-4}$
Control	30	3.00 ± 0.42 (0−9)	30	3.34 ± 0.31 (1.09−7.40)
Exposed	28	9.96 ± 1.02* (2−19)	27	5.03 ± 0.81* (0.84−17.11)

Note. Asterisk indicates significant difference ($p < .05$) with regard to the control group.

TABLE 3. Influence of Age and Sex on the Genotoxity Biomarkers Studied, With Models Adjusted by Exposure

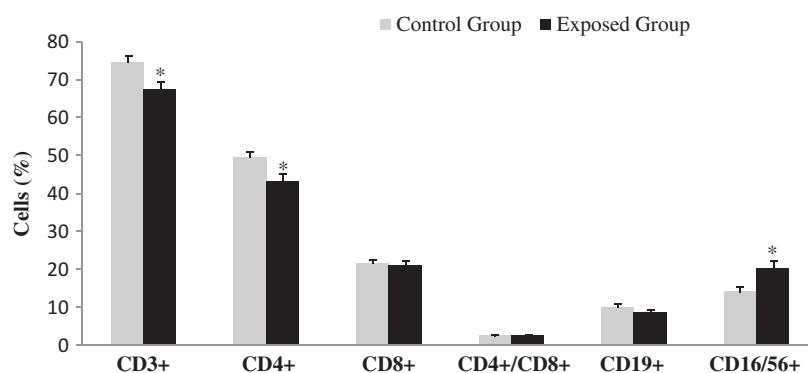| Model	Unstandardized coefficients β	95 % CI	Partial p value	R^2	Model p value
1. TCR-Mf					
Age(yr)	0.080	−0.003 to 0.162	.059	0.097	.024
Females vs. males	−0.891	−2.623 to 0.841	.307	0.054	.084
2. MN frequency					
Age(yr)	0.064	−0.046 to 0.174	.252	0.422	<.001
Females vs. males	3.268	1.183 to 5.353	.003	0.498	<.001

FIGURE 1. Differences in the percentages of lymphocyte subsets analysed in the study populations. Asterisk indicates significantly different from control, $p < .05$.

damage induced by chronic exposure to metal contamination. To our knowledge this is the first study in which TCR-Mf was evaluated in human populations exposed to As and Cd. It is well established that exposure to As and Cd produces inhibition of DNA repair mechanisms leading indirectly to increased mutations (Hartwig 1998; Hartwig and Schwerdtle 2002). This may explain the enhanced mutation frequency found in the current study.

Existing studies point to a rise in mutation frequency in each cell division due to errors associated with DNA replication. Akiyama et al. (1995) found a significant increase in TCR-Mf associated with age (increasing 2×10^{-5} for every 10 yr of age). Results of the multifactor analysis, adjusted by exposure, showed no significant influence of age on the TCR-Mf (Table 3). Nevertheless, a significant correlation was found between these two variables. Our data agree with previous studies reporting higher TCR-Mf in males than females (Kyoizumi et al. 1992; Akiyama et al. 1995), although this gender influence was not significant, as this may be attributed to a small population sample size.

Among the different genotoxicity biomarkers, MN test in peripheral blood lymphocytes was proposed as a useful tool to assess cytogenetic damage. One of the major advantages of the MN assay is that in addition to measuring clastogenic effects easily, the aneugenic effects can also be detected (Martinez et al. 2004). In our study significant differences

between exposed and controls were obtained in the MN test, with higher frequencies in the exposed population. This is in accordance with most studies that evaluated the effect of metals, particularly As and Cd, on MN frequency (Basu et al. 2002; Kašuba and Rozgaj 2002; Martinez et al. 2004). Furthermore, the significant correlation observed between TCR-Mf and MN frequency demonstrates a reliable synergy between the two genotoxicity biomarkers selected.

Several studies reported an association between gender and cytogenetic damage using MN frequency. Bonassi et al. (1995) found that the MN frequencies in females were 20–30% higher than males. Significant influence of gender, adjusted by exposure, on this cytogenetic rate was also demonstrated in this study, with females showing MN frequencies 38% higher than males (31% in control and 45% in exposed). Generally, the rise in MN frequencies observed in women was attributed to presumably preferential aneuploidogenic events involving the X chromosome (Barale et al. 1998). This chromosome is represented in MN more often than expected if equal probability between this sex chromosome and autosomes is presumed. Surrallés at al. (1996) reported an excessive overrepresentation of X chromosome in MN of lymphocytes cultured from women. The reason for decreased inclusion of autosomes and increased inclusion of X chromosome in MN of binucleated cells remains unclear.

It was suggested that a strong dependence seems to exist between MN frequencies and age (Peace and Succop 1999), and data demonstrating this relationship were reported by the HUman MicroNucleus (HUMN) international project (Bonassi et al. 2001). Analysis of our results did not show an influence of age on MN frequency. Nevertheless, a significant and positive correlation between these two variables was achieved, indicating the need to analyze a larger population in order to attain potential significance for this influence.

Hernandez-Castro et al. (2009) demonstrated that an increased, diminished, or absent function of immune-system cells is clearly associated with autoimmune diseases, deregulation of the immune response, and defective immune response against neoplastic cells or different pathogens. In order to determine whether this particular environmental exposure may involve some adverse effects on the immune system, variations in percentages of several lymphocyte subsets was determined. It is important to note that a homogeneous population was examined, as it is known that the number of lymphocytes vary depending on differing life situations (Moszczyński et al. 2001).

There is a substantial amount of information reported on the immunotoxic effects of metal exposure in animals (Cabassi 2007). With respect to humans such investigations are still lacking and most results are inconsistent. Decreases in %CD3$^+$ and %CD4$^+$ cells observed in our study may be related to the capacity of most metals, including As and Cd, to induce apoptosis of peripheral blood mononuclear cells (de la Fuente et al. 2002). Some of our results are also in accordance with a study performed by Soto-Peña et al. (2008) on the effects of As exposure in mice lymphocytes, which found reduced %CD4$^+$ cells but no marked effects on %CD8$^+$ and %CD19$^+$ cells. Soto-Pena et al. (2008) also reported a significant difference in %CD4$^+$/%CD8$^+$ ratio and no marked difference in %CD3$^+$ cells. In epidemiological terms, a change in a single immune test in an individual may not be a reliable biomarker of an increased susceptibility for disease; however, a variation in an immune biomarker in the entire population may be indicative of immunotoxicity (Moszczyński et al. 2001).

In our study significant influences were obtained in percent lymphocytes subsets for age and gender (data no shown). For %CD3$^+$ and %CD8$^+$ cells a significant decrease with age was found, whereas for %CD16/56$^+$ a significant increase was observed. With respect to gender, significantly higher values of %CD4$^+$ were obtained in women. These results are in accordance with data obtained in other studies, although some results are conflicting (Osugi et al. 1995; Shahabuddin et al. 1998; Rudy et al. 2002).

Thus, our results suggest that environmental exposure to metals derived from mining activities induce genotoxic effects as evidenced by significant increase in TCR-Mf and MN frequency, and by immunotoxic effects as noted by significant variation in percent lymphocytes subsets in the inhabitants of the surrounding mining area.

CONCLUSIONS

In conclusion, results of the present study indicate that the exposed population is experiencing genotoxic and immunotoxic effects originating from exposure to environmental contamination resulting from mining activities, since almost all studied biomarkers were significantly altered in exposed compared to a control population, even after adjustment for all the confounding factors. Genotoxic and immunotoxic effects may lead to several diseases including cancer. At this time one cannot postulate that observed effects are directly related to As and Cd exposure, as biomarkers of internal dose are lacking, even though our results are in accordance with the data observed for these metals published previously. Further studies with an increased number of individuals in each group and the integration of complementary biomarkers, namely, biomarkers of internal dose and susceptibility, are being planned that will enable us to better characterize the adverse health effects that are ongoing

in the study population with much higher statistical power and biological significance.

REFERENCES

Akiyama, M., Kyoizumi, S., Hirai, Y., Kusunoki, Y., Iwamoto, K. S., and Nakamura, N. 1995. Mutation frequency in human blood cells increases with age. *Mutat. Res*. 338: 141–49.

Ávila, P., Silva, E., Salgueiro, A., and Farinha, J. 2008. Geochemistry and mineralogy of mill tailings impoundments from the Panasqueira mine (Portugal): Implications for the surrounding environment. *Mine Water Environ*. 27: 210–24.

Balmain, A., Gray, J., and Ponder, B. A. 2003. The genetics and genomics of cancer. *Nat. Genet*. 33: 238–44.

Barale, R., Chelotti, L., Davini, T., Del Ry, S., Andreassi, M. G., Ballardin, M., Bulleri, M., He, J., Baldacci, S., Di Pede, F., Gemignani, F., and Landi, S. 1998. Sister chromatid exchange and micronucleus frequency in human lymphocytes of 1650 subjects in an Italian population: II. Contribution of sex, age, and lifestyle. *Environ. Mol. Mutagen*. 31: 228–42.

Basu, A., Mahata, J., Roy, A., Sarkar, J., Poddar, G., Nandy, A., Sarkar, P., Dutta, P., Banerjee, A., Das, M., Ray, K., Roychaudhury, S., Natarajan, A., Nilsson, R., and Giri, A. 2002. Enhanced frequency of micronuclei in individuals exposed to arsenic through drinking water in West Bengal, India. *Mutat. Res*. 516: 29–40.

Basu, A., Som, A., Ghoshal, S., Monday, L., Chaubey, R., Bhilwade, H., Rahman, M., and Giri, A. 2005. Assessment of DNA damage in peripheral blood lymphocytes of individuals susceptible to arsenic induced toxicity in West Bengal, India. *Toxicol. Lett*. 159: 100–12.

Bernstam, L., and Nriagu, J. 2000. Molecular aspects of arsenic stress. *J. Toxicol. Environ. Health B* 3: 293–322.

Bonassi, S., Bolognesi, C., Abbondandolo, A., Barale, R., Bigatti, P., Camurri, L., Dalpra, L., de Ferrari, M., Forni, A., and Lando, C. 1995. Infuence of sex on cytogenetic end points: Evidence from a large human sample and review of literature. *Cancer Epidemiol. Biomarkers Prev*. 4: 671–79.

Bonassi, S., Fenech, M., Lando, C., Lin, Y., Ceppi, M., Chang, W. P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Ferguson, L. R., Fucic, A., Lima, O. G., Hrelia, P., Krishnaja, A. P., Lee, T.-K., Migliore, L., Mikhalevich, L., Mirkova, E., Mosesso, P., Müller, W.-U., Odagiri, Y., Scarfi, M. R., Szabova, E., Vorobtsova, I., Vral, A., and Zijno, A. 2001. HUman MicroNucleus project: International database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *Environ. Mol. Mutagen*. 37: 31–45.

Cabassi, E. 2007. The immune system and exposure to xenobiotics in animals. *Vet. Res. Commun*. 31: 115–20.

Coelho, P., Silva, S., Roma-Torres, J., Costa, C., Henriques, A., Teixeira, J., Gomes, M., and. Mayan, O. 2007. Health impact of living near an abandoned mine—Case study: Jales Mines. *Int. J. Hyg. Environ. Health* 210: 399–402.

Costa, C., Teixeira, J.P., Silva S., Roma-Torres, J., Coelho, P., Gaspar, J., Alves, M., Laffon, B., Rueff, J., and Mayan, O. 2006. Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides. *Mutagenesis* 21: 343–50.

de La Fuente, H., Portales-Perez, D., Baranda, L., Diaz-Barriga, F., Saavedra-Alanís, V., Layseca, E., and González-Amaro, R. 2002. Effect of arsenic, cadmium and lead on the induction of apoptosis of normal human mononuclear cells. *Clin. Exp. Immunol*. 129: 69–77.

Department for Environment Food and Rural Affairs (DEFRA), and Environment Agency (EA). 2002. *Contaminants in soil: Collation of toxicological data and intake values for humans: Cadmium*. Report Tox 3. Bristol, UK: Environment Agency.

Dopp, E., Hartmann, L., Florea, A., von Recklinghausen, U., Pieper, R., Shokouhi, B., Rettenmeier, A., Hirner, A., and Obe, G. 2004. Uptake of inorganic and organic derivatives of arsenic associated with induced cytotoxic and genotoxic effects in Chinese hamster ovary (CHO) cells. *Toxicol. Appl. Pharmacol*. 201: 156–65.

Fenech, M. 2007. Cytokinesis-block micronucleus cytome assay. *Nat. Protocols* 2: 1084–104.

García-Lestón, J., Roma-Torres, J., Vilares, Maria, Rui Pinto, Cunha, L., Prista, João, Teixeira, J. P., Mayan, O., Pásaro, E., Méndez, J., and Laffon, B. 2011. Biomonitoring of a population of Portuguese workers exposed to lead. *Mutat. Res*. 721: 81–88.

Goering, P. L., Waalkes, M. P., and Klaassen, C. D. 1995. Toxicology of cadmium. In *Toxicology of metals: Biochemical aspects handbook of experimental pharmacology*, eds. R. A. Goyer and M. G. Cherian, Vol. 115, 189–213. New York: Springer-Verlag.

Gonsebatt, M., Vega, L., Montero, R., García, G., Del Razo, L., Albores, A., Cebrián, M., and Ostrosky-Wegman, P. 1994. Lymphocyte replication ability in individuals exposed to arsenic via drinking water. *Mutat. Res*. 313: 293–99.

Hartwig, A. 1998. Carcinogenicity of metal compounds: Possible role of DNA repair inhibition. *Toxicol. Lett*. 102–103: 235–2 39.

Hartwig, A., and Schwerdtle, T. 2002. Interactions by carcinogenic metal compounds with DNA repair processes: Toxicological implications. *Toxicol. Lett*. 127: 47–54.

Health Canada. 2004. *Canadian handbook on health impact assessment. A report of the Federal/Provincial/Territorial Committee on Environmental and Occupational Health*, Vol. 1. Ottawa: Health Canada.

Hernandez-Castro, B., Doniz-Padilla, L. M., Salgado-Bustamante, M., Rocha, D., Ortiz-Perez, M. D., Jimenez-Capdeville, M. E., Portales-Pérez, D. P., Quintanar-Stephano, A., and González-Amaro, R. 2009. Effect of arsenic on regulatory T cells. *J. Clin. Immunol*. 29: 461–69.

Heyworth, J. 1990. *Evaluation of the Port Pirie Environmental Health Program* (thesis). Adelaide (SA): Department of Community Medicine, University of Adelaide.

IARC. 1993. Cadmium and certain cadmium compounds. Beryllium, cadmium, mercury and exposures in the glass manufacturing industry. *IARC Monogr. Eval. Carcinogen. Risk Chem. Hum*. 58.

IARC. 2004. Some drinking-water disinfectants and contaminants, including arsenic. *IARC Monogr. Eval. Carcinogen. Risk Hum*. 84.

Jung, D., Bolm-Audroff, U., Faldum, A., Hengstler, J. G., Attia, D. I., Janssen, K., Reifenrath, M., Bienfait, H. G., Mayer-Popken, O., and Konietzko, J. 2003. Immunotoxicity of co-exposures to heavy metals: In vitro studies and results from occupational exposure to cadmium, cobalt and lead. *EXCLI J*. 2: 31–44.

Karakaya, A., Yucesoy, B., and Sardas, O. 1994. An immunological study on workers occupationally exposed to cadmium. *Hum. Exp. Toxicol*. 3: 73–75.

Kašuba, V., and Rozgaj, R. 2002. Micronucleus distribution in human peripheral blood lymphocytes treated in vitro with cadmium chloride in G_o and S phase of the cell cycle. *Chemosphere* 49: 91–95.

Kubota, M., Lin, Y.W., Hamahata, K., Sawada, M., Koishi, S., Hirota, H., and Wakazono, Y. 2000. Cancer chemotherapy and somatic cell mutation. *Mutat. Res*. 470: 93–102.

Kyoizumi, S., Umeki, S., Akiyama, M., Hirai, Y., Kusunoki, Y., Nakamura, N., Endoh, K., Konishi, J., Sasaki, M., Mori, T., Fujita, S., and Cologne, J. 1992. Frequency of mutant T lymphocytes defective in the expression of the T-cell antigen receptor gene among radiation-exposed people. *Mutat. Res*. 265: 173–80.

Ladou, J. 1995. A global ban on lead mining and primary smelting. *Int. J. Occup. Environ*. 1: 70–71.

Léonard, A., and Lauwerys, R. 1980. Carcinogenicity, teratogenicity and mutagenicity of arsenic. *Mutat. Res*. 75: 49–62.

Luebke, R. W., Chen, D. H., Dietert, R., Yang, Y., King, M., and Luster, M. I. 2006. The comparative immunotoxicity of five selected compounds following developmental or adult exposure. *J. Toxicol. Environ. Health B* 9: 1–26.

Martínez, V., Creus, A., Venegas, W., Arroyo, A., Beck, J. P., Gebel, T. W., Surrallés, J., and Marcos, R. 2004. Evaluation of micronucleus induction in a Chilean population environmentally exposed to arsenic. *Mutat. Res.* 564: 65–74.

Meng, Z., and Meng, N. 2000. Effects of arsenic on blast transformation and DNA synthesis of human blood lymphocytes. *Chemosphere* 41: 115–19.

Moszczyński., P, Zabiński, Z, Moszczyński, P. Jr., Rutowski, J., Słowiński, S., and, Tabarowski, Z. 2001. Immunological findings in cigarette smokers. *Toxicol. Lett.* 118: 121–27.

Mourón, S., Grillo, C., Dulot, F., and Golijow, C. 2006. Induction of DNA strand breaks, DNA-protein crooslinks and sister chromatid exchange by arsenite in a human lung cell line. *Toxicol. In Vitro* 20: 279–85.

Orloff, K., Mistry, K., and Metcalf, S. 2009 Biomonitoring for environmental exposures to arsenic. *J. Toxicol. Environ. Health B* 12: 509–24.

Osugi, Y., Hara, J., Kurahashi, H., Sakata, N., Inoue, M., Yumura-Yagi, K., Kawa-Ha, K., Okada, S., and Tawa, A. 1995. Age-related changes in surface antigens on peripheral lymphocytes of healthy children. *Clin. Exp. Immunol.* 100: 543–48.

Palus, J., Lewinska, D., Dziubaltowska, E., Stepnik, M., Beck, J., Rydzynski, K., and Nilsson, R. 2005. DNA damage in leucocytes of workers occupationally exposed to arsenic in copper smelters. *Environ. Mol. Mutagen.* 46: 81–87.

Peace, B. E., and Succop, P. 1999. Spontaneous micronucleus frequency and age: what are normal values? *Mutat. Res.* 425:225–30.

Reid, T., Feig, D., and Loeb, L. 1993. Mutagenesis by metal-induced oxygen radicals. *Environ. Health Perspect.* 102:57–61.

Rudy, B. J., Wilson, C. M., Durako, S., Moscicki, A. B., Muenz, L., and, Douglas, S. D. 2002.Peripheral blood lymphocyte subsets in adolescents: A longitudinal analysis from the REACH project. *Clin. Diagn. Lab. Immunol.* 9: 959–65.

Salgueiro, A., Avila, P., Pereira, H., and Silva, E. 2008. Assessment of the impact of pollution by arsenic in the vicinity of Panasqueira Mine (Portugal). *Proceedings of the geoENV 2008—7th International Conference on Geostatistics for Environmental Applications*, September 8–10, 2008, Southampton, UK.

Shahabuddin, S., Al-Ayedi, I., Gad El-Rab, M. O., and Qureshi, M. I. 1998. Age-related changes in blood lymphocyte subsets of Saudi Arabian healthy children. *Clin. Diagn. Lab. Immunol.* 5: 632–35.

Soto-Peña, G., Luna, A., Acosta-Saavedra, L., Conde-Moo, P., López-Carrillo, L., Cebrian, M., Bastida, M., Calderon-Aranda, E., and Vega, L. 2006. Assessment of lymphocyte supopulations and cytokine secretion in children exposed to arsenic. *FASEB J.* 20: 779–81.

Soto-Peña, G. A., and Vega, L. 2008. Arsenic interferes with the signaling transduction pathway of T cell receptor activation by increasing basal and induced phosphorylation of Lck and Fyn in spleen cells. *Toxicol. Appl. Pharmacol.* 230: 216–26.

Steinmaus, C., Moore, L. E., Shipp, M., Kalman, D., Rey, O. A., Biggs, M. L., Hopenhayn, C., Bates, M. N., Zheng, S., Wiencke, J. K., and Smith, A. H. 2007. Genetic polymorphisms in MTHFR 677 and 1298, GSTM1 and T1, and metabolism of arsenic. *J. Toxicol. Environ. Health A* 70: 159–70.

Stephens, C., and Ahern, M. 2001. *Worker and community health impacts related to mining operations internationally. A rapid review of the literature*. Paper prepared for MMSD. London: IIED and WBCSD.

Surrallés, J., Falck, G., and Norppa, H. 1996. In vivo cytogenetic damage revealed by FISH analysis of micronuclei in uncultured human T lymphocytes. *Cytogenet. Cell Genet.* 75: 151–54.

Taooka, Y., Takeichi, N., Noso, Y., Kawano, N, Apsalikov, K., and Hoshi, M. 2006.

Increased T-cell receptor mutation frequency in radiation-exposed residents living near the Semipalatinsk nuclear test site. *J. Radiat. Res*. 47: 179–81.

Tsai, S.-M., Wang, T.-N. and Ko, Y.-C. 1998. Cancer mortality trends in a blackfoot disease endemic community of Taiwan following water source replacement. *J. Toxicol. Environ. Health A* 55: 389–404.

U.S. Environmental Protection Agency. 1992. *Record of decision—Bunker Hill Mining and Metallurgical Complex, Soshone County, Idaho*. http://cfpub.epa.gov/superrods/index.cfm?fuseaction=data.siterods&siteid=1000195

Vega, L., Gonsebatt, M., and Ostrosky-Wegman, P. 1995. Aneugenic effect of sodium arsenite on human lymphocytes in vitro: An individual susceptibility effect detected. *Mutat. Res*. 334: 365–73.

Vega, L., Ostrosky-Wegman, P., Fortoul, T., Díaz, C., Madrid, V., and Saavedra, R. 1999. Sodium arsenite reduces proliferation of human activated T-cells by inhibition of the secretion of interleukin-2. *Immunopharmacol. Immunotoxicol*. 21: 203–20.

World Health Organization. 2004. *Water-related diseases. Arsenicosis*. Prepared for World Water Day 2001. Reviewed by staff and experts from the Programme for Promotion of Chemical Safety (PCS), and the Water, Sanitation and Health unit (WSH), World Health Organization (WHO), Geneva.

Yang, C.-Y., Chang, C.-C., Ho, S.-C. and Chiu, H.-F. 2008. Is colon cancer mortality related to arsenic exposure? *J. Toxicol. Environ. Health A* 71: 533–38.

Yucesoy, B., Turhan, A., Ure, M., Imir, T., and Karakaya A. 1997. Effects of occupational lead and cadmium exposure on some immunoregulatory cytokine levels in man. *Toxicology* 123: 143–47.

Metal(Loid) Levels in Biological Matrices from Human Populations Exposed to Mining Contamination—Panasqueira Mine (Portugal)

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METAL(LOID) LEVELS IN BIOLOGICAL MATRICES FROM HUMAN POPULATIONS EXPOSED TO MINING CONTAMINATION—PANASQUEIRA MINE (PORTUGAL)

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Mining activities may affect the health of miners and communities living near mining sites, and these health effects may persist even when the mine is abandoned. During mining processes various toxic wastes are produced and released into the surrounding environment, resulting in contamination of air, drinking water, rivers, plants, and soils. In a geochemical sampling campaign undertaken in the Panasqueira Mine area of central Portugal, an anomalous distribution of several metals and arsenic (As) was identified in various environmental media. Several potentially harmful elements, including As, cadmium (Cd), chromium (Cr), manganese (Mn), nickel (Ni), lead (Pb), and selenium (Se), were quantified in blood, urine, hair, and nails (toe and finger) from a group of individuals living near the Panasqueira Mine who were environmentally and occupationally exposed. A group with similar demographic characteristics without known exposure to mining activities was also compared. Genotoxicity was evaluated by means of T-cell receptor (TCR) mutation assay, and percentages of different lymphocyte subsets were selected as immunotoxicity biomarkers. Inductively coupled plasma–mass spectrometry (ICP-MS) and inductively coupled plasma–atomic emission spectrometry (ICP-AES) analysis showed elevated levels of As, Cd, Cr, Mn, and Pb in all biological samples taken from populations living close to the mine compared to controls. Genotoxic and immunotoxic differences were also observed. The results provide evidence of an elevated potential risk to the health of populations, with environmental and occupational exposures resulting from mining activities. Further, the results emphasize the need to implement preventive measures, remediation, and rehabilitation plans for the region.

Mining plays an important role in both local and national economies; however, in some locations the activity is still performed in an uncontrolled way, giving rise to soil erosion and serious environmental contamination (Coelho et al. 2007). Activities associated with mining are also hazardous activities, in both the environmental and public health contexts (Coelho et al. 2011b). Mining activities adversely affect the health of miners and the communities living near mine sites, and these effects may persist even after the mine is closed (Heyworth 1990;

Ladou 1995; U.S. EPA 1992). It is worth noting that many adverse effects are only observed in susceptible groups such as children and older people (Stephens and Ahern 2001). Further, chronic effects that might not be immediately apparent represent an important issue that also needs to be taken into account.

The choice of the Panasqueira Mine as a case study (Coelho et al. 2011a) was based on previous data from geochemical studies in this region that point to an anomalous distribution of arsenic (As) and other metals in the soil (Salgueiro et al. 2008) and also to an enrichment of As, cadmium (Cd), copper (Cu), and zinc (Zn) in sediments of local watercourses (Ávila et al. 2008). Considering that the populations in the area are highly dependent on land and water use for agriculture and cattle breeding, it is conceivable that the inhabitants are exposed and therefore adverse effects on health need to be investigated.

Certain metals are known to induce genotoxicity, and are known to produce cancer in a variety of animal species (Bernstam and Nriagu 2000; Chiu et al. 2004; Shi et al. 1999). Based on epidemiological data, As and Cd were classified as human carcinogens (Reid et al. 1993). Further, there is also a significant amount of data reported on the immunotoxic effects of metal(loid)s (Cabassi 2007; Luebke et al. 2006), although few investigations have been conducted in humans and the available results are controversial.

Information on the elemental content of human samples, such as liver, kidneys, brain, blood, and urine, is well established. Collection of the majority of these samples is invasive, and this limits their usage for biomonitoring studies. Obtaining nail and hair samples is a less invasive option and provides a number of advantages, such as (1) ease of collection, storage, and transportation; (2) possibility of monitoring elements accumulated over a period of a few weeks to a few months; (3) long-term sample stability; and (4) straightforward analysis using conventional elemental analysis methods such as inductively coupled plasma–mass spectrometry (ICP-MS) and inductively coupled plasma–atomic emission spectrometry (ICP-AES) (He 2011). These

techniques are the most commonly performed and routinely used for multielemental analysis of biological matrices (Caroli et al. 1992; Delves 1988; Taylor et al. 2011).

For more than three decades, hair and nail samples have been used to assess environmental and occupational exposure to metal(loid)s (Bencjo 1995; Bortoli et al. 1992; Brima et al. 2006; Button et al. 2009; Chatt et al. 1980; Creason et al. 1982; Kuangfei et al. 1999; Lin et al. 1998; Nowak and Kozlowski 1998; Orloff et al. 2009; Rodushkin et al. 2000). Several techniques are used, and Valkovic (1988) showed that for some elements data obtained were dependent on the method used. Data are usually compared to "normal" or reference concentration ranges for unexposed populations and for many elements the published ranges are fairly wide (Caroli et al. 1992; 1994; Katz and Chatt 1988; Miekeley et al. 1998; Valkovic 1988). This variation may be attributed to many factors that affect the element content in these matrices, such as age, gender, diet, lifestyle, and geochemical environment, among others (Rodushkin et al. 2000).

Our previous study at the Panasqueira Mine (Coelho et al. 2011a) indicated that the exposed population was experiencing genotoxic and immunotoxic effects derived from exposure to the environmental contamination resulting from mining activities; however, the limited number of samples, the lack of internal dose biomarkers, and also the fact that only environmental exposure was assessed limited any major conclusions. Considering all the points previously mentioned, the main objectives of the present study were to evaluate metal(loid)s contamination in environmentally and occupationally exposed subjects, and to relate this to genotoxic and immunotoxic effects. Concentrations of several metal(loid)s in blood, hair, fingernail, and toenail samples, quantified by ICP-MS and ICP-AES, were used as biomarkers of internal dose. Genotoxicity was evaluated by means of a T-cell receptor (TCR) mutation assay, and percentages of different lymphocyte subsets were selected as immunotoxicity biomarkers.

MATERIAL AND METHODS

Study Population

The study population consisted of a total of 102 subjects living in villages near the Panasqueira tungsten–tin mine site in central Portugal. The group consisted of 34 environmentally exposed individuals (10 men and 24 women), 33 occupationally exposed male individuals (workers from Panasqueira Mine), and 35 individuals without environmental or occupational exposure to mining activities who worked mainly in administrative offices and were matched with the first group by age, gender, lifestyle, and smoking habits (13 men and 22 women). The last group (control group) was located in a no-contaminated area upstream from the mine site. Individuals living in the villages for at least 5 yr and aged over 18 yr were selected. The characteristics of the groups are described in Table 1. Health conditions, medical history, medication, diagnostic tests (x-rays etc.), and lifestyle factors were assessed by means of questionnaires. Subjects also provided information with regard to specific symptoms related to metal(loid)s exposure and chronic respiratory diseases, such as bronchitis; drinking and agricultural water sources; agricultural practices, including pesticides usage; and diet. All subjects were fully informed about the procedures and objectives of this study, and signed an informed consent form prior to the study. Approval for this study was obtained from the Institutional Ethical Board of the Portuguese National Institute of Health.

Sample Collection

Blood samples were collected by venipuncture in BD Vacutainer CPT cell preparation tubes with sodium heparin (Becton Dickinson) for mononuclear leukocyte isolation prior to TCR mutation assay, and in sterile tubes containing ethylenediamine tetraacetic acid (EDTA) for metal(loid)s quantification and analysis of lymphocytes subsets. First morning urine samples (minimum volume: 20 ml) were collected in polyethylene sterile bottles. Hair and nail (finger and toe) samples were collected with stainless steel scissors and nail clippers and stored in polyethylene bags. Samples were transported under refrigeration and kept at approximately +4°C (blood) and –20°C (urine, hair, and nails) until analysis. All samples were coded and analyzed under blind conditions.

Metal(loid) Quantification

Instrumentation The ICP-MS instrument used were a PerkinElmer Elan DRC II, and an X Series Thermo Elemental. Perkin-Elmer Optima 5300 was used for ICP-AES. For microwave digestion an Anton Paar Multiwave 3000 oven equipped with 16 vessels was used. Before use and between each batch of samples, the polyether ether ketone (PEEK) utensils were thoroughly acid washed and then rinsed with deionized water.

Reagents and Standards

All reagents used were trace analysis grade or better quality. All aqueous solutions were

TABLE 1. Characteristics of the Study Population

	Control group	Environ. exposed group	Occup. exposed group
Number of subjects	35	34	33
Age (yr)	58 ± 11	62 ± 14	62 ± 8
(mean ± SD; range)	(42–80)	(22–80)	(51–81)
Gender	22 Females	24 Females	33 Males
	13 Males	10 Males	
Smoking habits			
Nonsmokers, n (%)	30 (86%)	31 (91%)	26 (79%)
Smokers, n (%)	5 (14%)	3 (9%)	7 (21%)

Note. SD, Standard deviaton.

prepared using ultrapure water ($>18 \ \Omega \ cm^{-1}$). For analysis of blood samples In and Sc were the chemical elements used as internal standards while Ge and Ir were used for urine, hair, and nail samples.

Sample Preparation   Blood samples (1–2 g) were added to 2 ml HNO_3 in Teflon vials and digested for 24 h at 100°C. After cooling, the digested solutions were diluted with deionized water up to 10 ml in polypropylene tubes for elemental analysis by ICP-MS and ICP-AES. Urine samples were defrosted 24 h before the analysis, centrifuged for 3 min at 2000 × g, and diluted up to 25-fold with 1% v/v HNO_3 for elemental analysis by ICP-MS. Hair samples were washed following a slightly modified version of the protocol described by Schrauzer et al. (1992), which involved washing the samples 4 times with 1% Triton X-100, rinsing once with acetone, thrice with deionized water, and twice more with acetone. Each sample was washed for approximately 30 min (total time), and subsequently all the samples were dried in an oven at 70°C for 2 h. Fingernail and toenail samples were washed thoroughly following a slightly modified version of the protocol described by Slotnick et al. (2007). Visible exogenous material was first removed using plastic forceps. Samples were then placed in clean glass vials and sonicated for 5 min using acetone, rinsed with deionized water followed by acetone, sonicated for 10 min with deionized water, then rinsed twice with deionized water, ensuring complete submersion of the sample during each step. All samples were oven-dried at 60°C overnight.

Hair, fingernail, and toenail samples were acid digested using closed vessel microwave-assisted heating. Into each vessel 2 ml HNO_3 was added to samples and left to stand for 1 h before sealing the vessels. The average sample mass for hair, fingernail, and toenail samples was, respectively, 207.6 ± 8.1 mg, 37.8 ± 3.6 mg, and 59.7 ± 6.5 mg. The microwave heating program was: 100% power (1200 W), 15 min ramp to 170°C, held for 10 min, then held for 20 min at room temperature. The pressure in the system was approximately 200 psi under these conditions. This method resulted in complete sample dissolution. The solutions were transferred to plastic tubes and made up to 5 ml with ultrapure water for direct determination via ICP-MS.

Quality Control and Quality Assurance

Blood analyses were validated using European Union, Institute for Reference Materials and Measurements—Certified Reference Materials (CRM) BCR 634, BCR 635, and BCR 636. Recoveries varied between 81 and 83% and the precision of the method (for Cd and Pb) was better than 10% coefficient of variation.

Freeze-dried human urine CRM from National Institute of Environmental Studies (NIES), Japan, was used in addition to validating the metal quantification in urine samples; this material was also analyzed during each analytical run as a quality control (QC) sample. Results from CRM were within the reference range for As, Pb, and Se.

As no nail CRM are currently available, the methods for digestion and analysis of hair, fingernail, and toenail samples were validated using a human hair CRM—NCS DC 73347a and NCS ZC 81002b human hair (NCS Beijing, China). The CRM were also used as QC standard by digesting a portion with each batch of samples and determining the metal concentrations—As, Cd, Cr, Mn, Ni, Pb, and Se—along with the other digests. Results from CRM were within the reference range. Other QC measures used in the different matrices included the periodic analysis of suitable standards to check on instrument drift and short-term stability.

Determination of Creatinine

Creatinine adjustment is routinely used to reduce some factors that are not related to metal(loid) exposure, such as urine concentration and urine volume (Hinwood et al. 2002). Results of urine samples were adjusted and reported as micrograms metal per gram creatinine. Creatinine was measured photometrically using the Jaffe reaction (Roche Diagnostics).

Genotoxicity and Immunotoxicity Assays

T-Cell Receptor Mutation Assay The T-cell receptor (TCR) mutation assay was carried out using a flow cytometric method. Mononuclear leukocytes suspensions were prepared following the protocol described by García-Lestón et al. (2010), and subsequently analyzed by a FACScalibur flow cytometer with Cell Quest Pro software (Becton Dickinson). At a minimum, 2.5×10^5 lymphocyte-gated events were acquired, and mutation frequencies of TCR (TCR-Mf) were calculated as the number of events in the mutant cell window (CD3$^-$CD4$^+$ cells) divided by the total number of events corresponding to CD4$^+$ cells.

Analysis of Lymphocytes Subsets The percentages of different lymphocyte subsets, namely, T lymphocytes (CD3$^+$ lymphocytes), T helper (Th) lymphocytes (CD4$^+$ lymphocytes), T cytotoxic (Tc) lymphocytes (CD8$^+$ lymphocytes), B lymphocytes (CD19$^+$ lymphocytes), and natural killer (NK) cells (CD16$^+$ and CD56$^+$ lymphocytes), were evaluated by flow cytometry as described in García-Lestón et al. (2010). Analysis was performed on a FACScalibur flow cytometer using Cell Quest Pro software (Becton Dickinson). At least 10^4 events in the lymphocytes window were acquired.

Statistical Analysis

Student's t-test and analysis of variance (ANOVA) were applied to assess statistical differences between means. Fisher's exact test for homogeneity or independence was applied to assess the relationship between categorical variables. Multifactorial analysis was performed to evaluate the contribution of potential confounding factors (age, gender and smoking habits) to the response variables considered. Pearson's correlation analysis was used for the estimation of correlations between parameters. The level of significance was set at $p < .05$. All statistical analyses were performed with the software SPSS 17.0 for Windows statistical package.

RESULTS

The general characteristics of the studied populations are summarized in Table 1. No significant differences in age and smoking habits between the three groups were observed. Gender difference between environmental and control group was not significant, the occupational group consisted only of males.

Metal quantification showed significant differences in the exposed populations for both the environmental and occupational groups compared to controls, for several elements in different matrices, namely, As in blood and nails, Cd in hair, Mn in hair, Pb in blood and hair, and Se in fingernails (Tables 2 to 6).

Results of the multifactor analysis, adjusted by exposure, demonstrated significant influence of age on (1) As and Se in urine, with higher concentrations in older individuals, (2) Ni and Pb in fingernails, with higher concentration in younger individuals, (3) significant influence of gender on Pb in blood, with higher concentration in males, and (4) no significant influence of smoking habit. Concerning age, significant correlations were found for As and Se in urine samples, and for Ni and Pb in fingernails with higher concentrations of these elements in older individuals. In addition, there were several significant correlations between different elements within the same matrix, different elements in different matrices, and for the same element in different matrices (see supplementary material, Tables 7–10 values presented are Person correlation coefficient and p value).

Analysis of TCR-mutation frequency (TCR-Mf) (Figure 1) showed significant differences between populations, with higher values in occupationally exposed individuals compared to controls. Results of the multifactor analysis, adjusted by exposure, demonstrated no significant influence of age, gender, or smoking habits on the TCR-Mf. However, a significant correlation was achieved between age and the genotoxicity biomarker evaluated.

Figure 2 shows the variation in the different lymphocyte subsets analyzed. Environmentally exposed individuals presented significantly lower %CD3$^+$ and %CD8$^+$

TABLE 2. Metal(loid)s Levels in Whole Blood Samples (μg/L)

	As	Cd	Mn	Pb	Se
Control group	11.76 ± 1.13	2.38 ± 0.19	21.28 ± 3.97	35.63 ± 4.11	200.67 ± 7.14
Environmental exposed group	16.46 ± 1.67*	2.28 ± 0.07	22.02 ± 1.15	35.99 ± 4.56	192.37 ± 6.27
Occupational exposed group	11.61 ± 2.08	2.82 ± 0.40	21.29 ± 5.01	60.48 ± 10.99**	192.18 ± 15.35
Reference range[a]	<10	<1, Nonsmokers <3, Smokers	4–12	<100	82–180

Note. Data are mean ± SEM. Significant differences indicated by: *$p < .05$, compared to controls; **$p < .05$, compared to controls and environmental exposed group.

[a]http://www.toxlab.co.uk/traceele.htm

TABLE 3. Metal(loid)s Levels in Urine Samples (μg/g creatinine)

	As	Cd	Cr	Mn	Ni	Pb	Se
Control group	67.46 ± 13.44	0.50 ± 0.06	1.58 ± 0.32	1.62 ± 0.42	6.39 ± 0.60	2.49 ± 0.41	29.39 ± 2.01
Environmental exposed group	51.01 ± 8.17	0.49 ± 0.06	1.58 ± 0.23	2.18 ± 0.33	5.92 ± 0.66	4.05 ± 1.69	28.35 ± 2.23
Occupational exposed group	43.01 ± 7.35	0.83 ± 0.35	1.15 ± 0.13	1.36 ± 0.27	8.16 ± 1.17	4.54 ± 1.33	31.15 ± 3.59
Reference range[a]	<10	<2	<1	<1	<5	<10	<30

Note. Data given as mean ± standard error.

[a]http://www.toxlab.co.uk/traceele.htm

TABLE 4. Metal(loid)s Levels in Fingernail Samples (μg/g)

	As	Cd	Cr	Mn	Ni	Pb	Se
Control group	0.11 ± 0.02	0.08 ± 0.02	1.10 ± 0.34	1.66 ± 0.60	2.43 ± 0.75	0.86 ± 0.17	0.66 ± 0.09
Environmental exposed group	0.35 ± 0.10*	0.12 ± 0.05	1.19 ± 0.55	1.59 ± 0.37	1.43 ± 0.28	1.05 ± 0.31	0.79 ± 0.14**
Occupational exposed group	1.19 ± 0.47*	0.08 ± 0.03	0.59 ± 0.16	1.83 ± 0.29	1.17 ± 0.28	1.38 ± 0.24	0.48 ± 0.04
Published ranges[a]	0.07–1.09	0.01–0.44	0.22–3.20	0.19–3.30	0.14–6.95	0.27–4.75	0.62–1.53

Note. Data given as mean ± standard error. Significant differences indicated by: *$p < .05$, compared to controls; **$p < .05$, compared to occupational-exposed group.

[a]Rodushkin et al. (2000).

TABLE 5. Metal(loid)s Levels in Toenail Samples (μg/g)

	As	Cd	Cr	Mn	Ni	Pb	Se
Control group	0.22 ± 0.06	0.03 ± 0.01	1.41 ± 0.30	1.30 ± 0.24	1.88 ± 0.54	1.09 ± 0.21	0.52 ± 0.04
Environmental exposed group	0.50 ± 0.09*	0.03 ± 0.01	1.76 ± 0.39	2.02 ± 0.49	1.73 ± 1.06	1.11 ± 0.34	0.60 ± 0.11
Occupational exposed group	0.75 ± 0.35	0.08 ± 0.04	1.71 ± 0.71	2.51 ± 0.70	1.85 ± 0.98	1.54 ± 0.54	0.60 ± 0.11
Published ranges[a]	0.07–1.09	0.01–0.44	0.22–3.20	0.19–3.30	0.14–6.95	0.27–4.75	0.62–1.53

Note. Data given as mean ± standard error. Significant differences indicated by *$p < .05$, compared to controls.

[a]Rodushkin et al. (2000).

cells and significantly higher %CD4+%CD8+ ratio and %CD16/56+ cells than controls. Occupationally exposed individuals showed significantly lower %CD3+ and %CD4+ cells and significantly higher %CD16/56+ cells than controls. In addition, these individuals

TABLE 6. Metal(loid)s Levels in Hair Samples (μg/g)

	As	Cd	Cr	Mn	Ni	Pb	Se
Control group	0.12 ± 0.02	0.04 ± 0.01	0.07 ± 0.01	0.86 ± 0.19	0.39 ± 0.06	1.74 ± 0.56	0.70 ± 0.21
Environmental exposed group	0.13 ± 0.03	0.05 ± 0.01	0.14 ± 0.05	0.69 ± 0.11	0.25 ± 0.04	1.33 ± 0.28	0.76 ± 0.30
Occupational exposed group	0.52 ± 0.23	$0.15 \pm 0.05^*$	0.19 ± 0.07	$1.58 \pm 0.30^{**}$	0.45 ± 0.10	$3.20 \pm 0.56^{**}$	0.50 ± 0.05
Published ranges[a]	0.03–0.32	0.01–0.36	0.05–0.53	0.08–2.41	0.11–1.60	0.22–7.26	0.48–1.84

Note. Data given as mean $\pm$ standard error. Signficant differences indicated by: $^*p < .05$, compared to controls; $^{**}p < .05$, compared to environmental-exposed group.
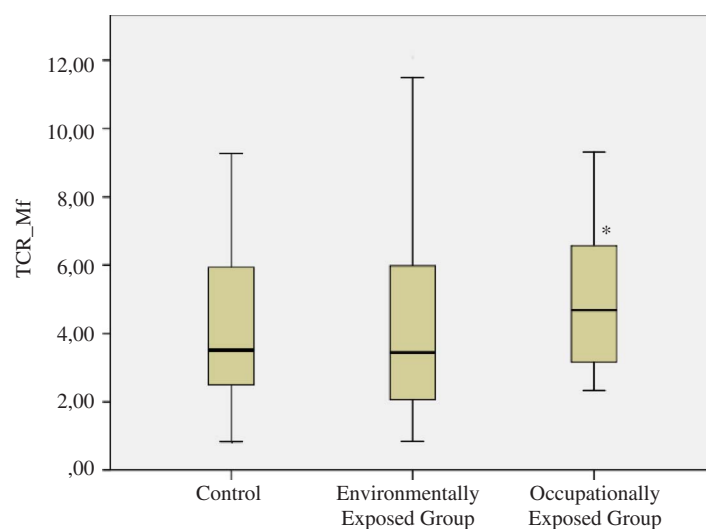
[a]Rodushkin et al. (2000).

FIGURE 1. Graphic representation of the TCR-Mf in the study populations. Asterisk indicates significantly different from control group ($p < .05$), according to independent samples t-test (color figure available online).
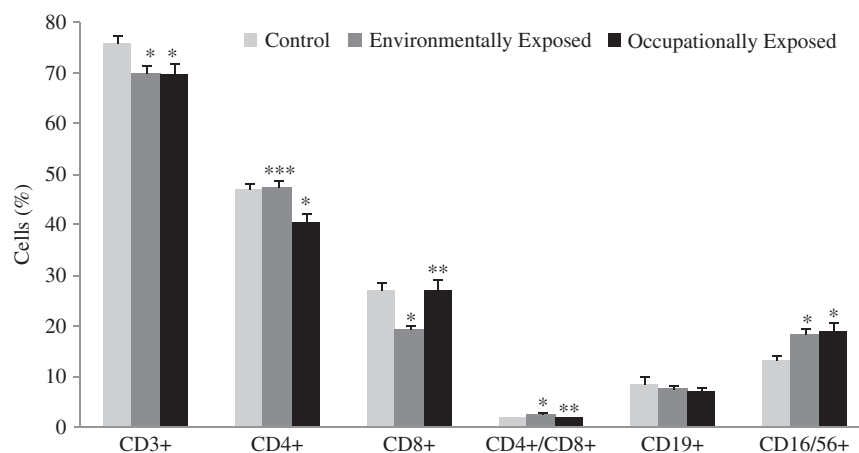

FIGURE 2. Differences in the percentages of lymphocyte subsets analyzed in the study populations. Asterisk, significantly different from control group; double asterisk significantly different from the environmentally exposed group ($p < .05$); triple asterisk, significantly different from the occupationally exposed group ($p < .05$), according to independent-samples t-test.

TABLE 7. Correlations Between Metal(loid)s in Blood and the Other Variables

	Mn B	Pb B	Se B	Cd U	Hg U	Ni U	Pb U	Se U	As TN	Cd TN	Hg TN	Se TN	As H	Cr H	Hg H	Se H	%CD16/56+
As B	0.382** 0.000		0.426** 0.000		0.211* 0.038							0.353** 0.001					0.287** 0.003
Cd B		0.476** 0.003		0.611** 0.000		0.496** 0.005										0.428* 0.011	
Mn B		0.226* 0.022	0.226* 0.023														
Pb B							0.274** 0.007										
Se B						−0.352** 0.000		−0.221* 0.029	−0.247* 0.019	−0.264* 0.023	0.218* 0.040		−0.302** 0.002	−0.295** 0.005	0.216* 0.030		

Note. B, blood; U, urine; TN, toenails; H, hair. *p < 0.05, **p < 0.01

TABLE 8. Correlations Between Metal(loid)s in Urine and the Other Variables

	Hg U	Mn U	Ni U	Pb U	Se U	Cr FN	Hg FN	Se FN	Cd TN	Cr TN	Hg TN	Pb TN	As H	Pb H	%CD4+
As U													0.278** 0.006		
Cd U			0.230* 0.023	0.589** 0.000											
Cr U	0.438** 0.000	0.519** 0.000	0.324** 0.001		0.305** 0.003	0.399** 0.001	0.266* 0.012	0.314** 0.002						−0.209* 0.047	
Hg U		0.267** 0.009	0.253* 0.013		0.350** 0.000	0.385** 0.001	0.563** 0.000	0.611** 0.000							
Mn U			0.215* 0.037		0.270** 0.008										0.264** 0.010
Ni U				0.227* 0.025	0.469** 0.000							0.231* 0.041			
Se U									0.372** 0.001	0.371** 0.000	0.308** 0.003	0.287* 0.010			

Note. U, urine; FN, fingernails; TN, toenails; H, hair. *p < 0.05, **p < 0.01

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TABLE 9. Correlations Between Metal(loid)s in Nails and the Other Variables

	CdFN	HgFN	NiFN	SeFN	AsTN	CdTN	CrTN	HgTN	MnTN	NiTN	PbTN	SeTN	AsH	HgH	NiH	PbH	SeH	%CD3+	%CD4+	%CD8+	%CD19+	%CD4/CD8+	%CD16/56+
As FN	0.281* 0.016				0.850** 0.000	0.436** 0.000							0.750** 0.000						−0.215* 0.043				
Cd FN					0.278* 0.019																		
Cr FN		0.549** 0.000	0.282* 0.034	0.640** 0.000																			
Hg FN			0.285* 0.018	0.681** 0.000							0.232* 0.047												
Mn FN																	0.425** 0.000	−0.213* 0.047					0.213* 0.046
Ni FN				0.374** 0.001										0.247* 0.038				0.238* 0.044					−0.275* 0.019
Pb FN						0.334* 0.016	0.426** 0.001	0.458** 0.000		0.322* 0.015	0.557** 0.000												
Se FN											0.232* 0.042					−0.210* 0.047				−0.212* 0.040	0.262* 0.011	0.203* 0.050	
As TN						0.431** 0.000			0.714** 0.000				0.718** 0.000										
Cd TN							0.551** 0.000	0.643** 0.000	0.277* 0.018	0.413** 0.000	0.527** 0.000	0.497** 0.000	0.610** 0.000										
Cr TN								0.685** 0.000	0.313** 0.003	0.582** 0.000	0.714** 0.000	0.587** 0.000											
Hg TN									0.229* 0.032	0.567** 0.000	0.678** 0.000	0.516** 0.000											
Mn TN											0.311** 0.006	0.247* 0.022	0.457** 0.000					−0.362** 0.000	−0.229* 0.030		0.223* 0.035		
Ni TN											0.488** 0.000	0.542** 0.000											
Pb TN												0.450** 0.000			0.343** 0.002								

Note. FN, fingernails; TN, toenails; H, hair. $*p < 0.05$, $**p < 0.01$

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TABLE 10. Correlations Between Metal(loid)s in Hair and the Other Variables

	Cd H	Hg H	Mn H	Ni H	Pb H	TCR-Mf	%CD4+	%CD8+	%CD4%CD8+
As H	0.238* 0.025		0.461** 0.000		0.302** 0.003				
Cd H		0.237* 0.025	0.492** 0.000		0.571** 0.000			0.281** 0.007	−0.213* 0.044
Cr H				0.560** 0.000		0.313** 0.003			
Hg H			0.258** 0.009						
Mn H				0.226* 0.029	0.552** 0.000				
Ni H							−0.322** 0.001	0.241* 0.019	

Note. H, hair. *p < 0.05, **p < 0.01

TABLE 11. Correlations Between TCR-mf and the Percentage of Lymphocyte Subset

	%CD4+	%CD8+	%CD19+	%CD4%CD8+	%CD16/56+
TCR-Mf	−0.217* 0.030				
%CD3+	0.480** 0.000	0.576** 0.000	−0.359** 0.000		−0.717** 0.000
%CD4+		−0.381** 0.000		0.610** 0.000	−0.309** 0.002
%CD8+			−0.259** 0.009	−0.750** 0.000	−0.447** 0.000
%CD19+					−0.195* 0.050

also demonstrated significantly lower %CD4+ cells and %CD4+%CD8+ ratio and significantly higher %CD8+ cells than those with environmental exposure. The other immunotoxicity parameter assessed (%CD19+ cells) was not significantly modified by exposure.

Results of the multifactor analysis, adjusted by exposure, showed significant influence of age on %CD3+ and %CD16/56+ cells, significant effect of gender on %CD19+, and no significant effect of smoking habits. Significant correlations were found between age and %CD3+ and %CD16/56+ cells. Significant correlations were also found among the majority of the lymphocyte subsets (see supplementary material). Finally, associations between different biomarkers were also analyzed and significant correlations were noted between TCR-Mf and Cr in hair samples, TCR-Mf and %CD4+, and several significant correlations between metal(loid)s concentrations in different matrices and immunotoxicity biomarkers.

DISCUSSION

As the results obtained in the geochemical analyses in the Panasqueira Mine area (Salgueiro et al. 2008; Ávila et al. 2008) showed an elevated contamination of stream water, sediments, and soils by metal(loid)s, many of which are human carcinogens known to induce genotoxicity and immunotoxicity, our main goal was to evaluate actual human exposure, and consequent genotoxic and immunotoxic effects. Most elements were at higher concentrations in exposed populations compared to controls. This was seen for all elements except for As in urine, Cr in urine, Ni in fingernails, and Se in blood (Tables 2 to 6).

There are few studies that report metal(loid)s quantification in the Portuguese population. Coelho et al. (2007) determined the concentration of Pb and Cd in a population exposed to mining residues in the North of Portugal (Jales Mines). Results obtained for Cd

were lower than those reported here for control and exposed populations, while Pb values were higher than found in the current study. In addition, the values reported by Reis et al. (2007) for Pb and Cd in blood from a population living close to Lisbon and another from Madeira were markedly different from each other and from those in this study. This reinforces the notion that metal(loid) levels change within the same population (Portuguese) in different geographic areas and therefore the choice of a reference value is even more difficult. Therefore, in this study results obtained were compared for the exposed population with those of controls, as these are matched for age, gender, diet, lifestyle, geochemical environment, and residence site. The results may also be compared with published reference ranges (Caroli et al. 1992; 1994; Katz and Chatt 1988; Miekeley et al. 1998; Valkovic 1988), but these ranges change significantly from population to population. Numerous factors such as residence site, gender, age, diet, lifestyle, and geochemical environment need to be taken into account when establishing the reference ranges for a population (Rodushkin et al. 2000). Therefore, such comparisons need to be interpreted carefully. For most elements studied the concentrations reported here were above the reference ranges for the English population in blood and urine samples (http://www.toxlab.co.uk/traceele.htm). Arsenic in fingernails and hair exceeded the published ranges for the Swedish population (nail and hair samples) (Rodushkin et al 2000).

Taking into account all these factors, one can state that both environmentally and occupationally exposed populations experienced a continuous exposure to As, as the values found in blood and nail samples were significantly higher compared to controls. Concerning Cd, Mn, and Pb, the occupationally exposed population seems to have experienced exposure to these elements in the past (few weeks to few months) but not in the present (few days), as significant results were obtained in hair samples but not in blood or urine. This highlights the importance of collecting and

analyzing different sample types to investigate exposure time course.

The significant difference obtained for Pb in blood between controls and occupationally exposed individuals was due to gender differences. After adjustment for this factor the difference was no longer significant. For Mn and Se the same picture was reflected, as shown with the concentrations of Mn in hair samples and Se in fingernails, with a significant difference for Mn in fingernails between controls and occupational exposed individuals noted after gender adjustment. Finally, no significant differences among groups were observed in any of the matrices for Cr, Ni, and Se, indicating no significant exposure to these elements due to the mining activity.

Several significant correlations were found between different elements within the same matrix, different elements in different matrices, and the same element in different matrices, demonstrating synergy between these biomarkers and the probability of a certain grade of similitude among the toxicokinetic mechanisms of the elements analyzed.

The significant increase in TCR-Mf found in this study in the environmentally (4.71 vs. 4) and occupationally exposed populations (5.95 vs. 4), compared to the control population, indicate mutagenic damage induced by chronic exposure to metal(loid) contamination. To our knowledge this is the first study in which TCR-Mf was evaluated in human populations exposed to metal(loid)s both environmentally and occupationally. Garcia-Lestón et al. (2010) evaluated this biomarker in a population occupationally exposed to Pb and also found significant differences between controls and exposed individuals. It is well established that exposure to these elements produces inhibition of DNA repair mechanisms leading indirectly to increased mutations (Hartwig 1998; Hartwig & Schwerdtle 2002). This may explain the increased mutation frequency found in the current study.

Existing studies also point to an increase in mutation frequency in each cell division, due to errors associated with DNA replication.

Indeed, Akiyama et al. (1995) found a significant increase in TCR-Mf associated with age (increasing 2×10^{-5} for every 10 yr of age). Results of the multifactor analysis, adjusted by exposure, showed no significant influence of age on the TCR-Mf. However, a significant correlation was found between these two variables.

Decreases in %CD3$^+$ and %CD4$^+$ cells observed in our study may be related to the capacity of most metal(loid)s to induce apoptosis of peripheral blood mononuclear cells (de la Fuente et al. 2002). Some of our results are also consistent with those of Soto-Peña and Vega (2008) on the effects of As exposure on mice lymphocytes and with those of Garcia-Lestón et al. (2010) on the effect of Pb in Portuguese factory workers. The former study reported reduced %CD4$^+$ cells, and %CD4$^+$/%CD8$^+$ ratio, similar to our data, and no effect in %CD19$^+$ cells, although no significant difference in %CD8$^+$ and %CD3$^+$ cells was found. As for the second study, a significant decrease in %CD8$^+$ cells and a significant increase in the %CD4$^+$/ %CD8$^+$ ratio was detected. Our results are also in agreement with Schwenk et al. (2008) for the effects of Cd, Cr, Ni, and Pb on lymphocyte subsets. With respect to %CD16/56$^+$ cells, studies are scarce and results divergent. In epidemiological terms, a change in a single immune test in an individual may not point to an increased susceptibility for disease, although a slight variation in an immune biomarker in the entire population may point to immunotoxicity (Moszczyński et al. 2001).

In our study significant influences of age and gender were seen in percentages of lymphocyte subsets. For %CD3$^+$ cells a significant decrease with age was noted, whereas for %CD16/56$^+$ cells a significant increase was observed. With gender, significantly higher values of %CD19$^+$ cells were obtained in females, which is in agreement with results obtained in other studies, although there are some conflicting results (Osugi et al. 1995; Rudy et al. 2002; Shahabuddin et al. 1998).

The only significant correlation found between TCR-Mf and any metal was for Cr in hair. Despite the fact that the difference between controls and exposed individuals did not reach significance for this element in this matrix, considerably higher concentrations for both exposed groups were obtained. This might indicate that the increase in the mutation frequency in the exposed populations may be related to exposure to this element. The observed significant negative correlation between TCR-Mf and %CD4$^+$ cells is supported by Tanigawa et al. (2008), who reported a decrease in %CD4$^+$ cells in human populations exposed to Cr. This suggests that exposure to this element induces mutations in the TCR genes which might negatively influence the %CD4$^+$ cells.

Finally the diverse and significant correlations found between percentages of different lymphocyte subsets and metal(loid) in different matrices, especially those between As and %CD16/56$^+$, Cd and %CD8$^+$ cells and %CD4$^+$/%CD8$^+$ ratio, Cr and %CD3$^+$ and %CD4$^+$ cells, and Mn and %CD3$^+$cells, reinforce the link between the medium- to long-term exposure to these elements and immunotoxic effects observed in exposed populations.

Our results show high concentrations of metal(loid)s in the biological matrices analyzed due to exposure to mining residues. This exposure leads to the induction of genotoxic effects and immunotoxic effects with significant increases in TCR-Mf and significant variation in percentages of lymphocyte subsets in miners and inhabitants of the area surrounding the mine complex.

CONCLUSIONS

In conclusion, results of the present study indicate that the populations studied are exposed, both environmentally and occupationally, to high concentrations of metal(loid)s, particularly As, Cd, Cr, Mn, and Pb, which lead to genotoxic and immunotoxic effects, since almost all studied biomarkers were significantly altered compared to control population, even after adjustment for common confounding factors. Genotoxic and

immunotoxic effects may result in several diseases, with cancer being of most concern. Results from this study are of paramount importance not only for these particular populations but to others exposed to similar conditions. It is crucial to conduct regular monitoring and surveillance activities of the environment, miners, and the community's health. Understanding the relationship between exposure and biological effect enables the development of preventive measures, remediation, and rehabilitation plans, thus leading to a reduction in cancer risks for humans. Further studies with the integration of complementary biomarkers, namely, other biomarkers of genotoxicity and susceptibility, and metal(loid)s speciation are being planned. This will enable us to reinforce the characterization of the adverse health effects that are ongoing in the study populations.

REFERENCES

Akiyama, M., Kyoizumi, S., Hirai, Y., Kusunoki, Y., Iwamoto, K. S., and Nakamura, N. 1995. Mutation frequency in human blood cells increases with age. *Mutat. Res*. 338: 141–49.

Avila, P. Silva, E., Salgueiro, A. and Farinha, J. 2008. Geochemistry and mineralogy of mill tailings impoundments from the Panasqueira mine (Portugal): Implications for the surrounding environment. *Mine Water Environ*. 27: 210–24.

Balmain, A., Gray, J., and Ponder, B. A. 2003. The genetics and genomics of cancer. *Nat. Genet*. 33: 238–44.

Bencjo, V. 1995. Use of human hair as a biomarker in the assessment of exposure to pollutants in occupational and environmental settings. *Toxicology* 101:29–39.

Bernstam, L. and Nriagu, J. 2000. Molecular aspects of arsenic stress. *J. Toxicol. Environ. Health B* 3: 293–322.

Bortoli, A., Gerotto, M., Marchiori, M., Palonta, R., and Troncon, A. 1992. Applications of inductively coupled plasma mass spectrometry to the early detection of potentially toxic elements. *Microchem. J*. 46:167–73.

Brima, E., Haris, P., Jenkins, R., Polya, D., Gault, A., and Harrington, C. 2006. Understanding arsenic metabolism through a comperative study of arsenic levels in the urine, hair and fingernails of healthy volunteers from three unexposed ethnic groups in the United Kingdom. *Toxicol. Appl. Pharmacol*. 216: 122–30.

Button, M., Jenkin, G., Harrington, C., and Watts, M. 2009. Human toenails as a biomarker of exposure to evevated environmental arsenic. *J. Environ. Monit*. 11: 610–17.

Cabassi, E. 2007. The immune system and exposure to xenobiotics in animals. *Vet. Res. Commun*. 31: 115–20.

Caroli, S., Senofonte, O., Violante, N., Fornarelli, L., and Powar, A.1992. Assessment of reference values for elements in hair of urban normal subjects. *Michrochem. J*. 46: 174–83.

Caroli, S., Alimonti, A., Coni, E., Petrucci, F., Senofonte, N. and Violante, N. 1994. The assessment of reference values for elements in human biological tissues and fluids: A systematic review. *Crit. Rev. Anal. Chem*. 24: 363–98.

Chatt, A., Secord, C., Tiefenbach, B., and Jervis, R. 1980. Scalp hair as a monitor of community exposure to environmental pollutants. In *Hair, trace elements and human illness*, eds. A. C. Brown and R. G. Crounce, 46–73. New York, NY: Praeger.

Chiu, H.-F., Ho, S.-C., Wang, L.-Y., Wu, T.-N., and Yang, C.-Y. 2004. Does arsenic exposure increase the risk of liver cancer? *J. Toxicol. Environ Health A* 67: 1491–1500.

Coelho, P., García-Lestón, J., Silva, S., Costa, C., Costa, S., Coelho, M., Lage, B., Mendez, E., and Teixeira, J. 2011a. Geno- and Immunotoxic effects on populations living near a mine: A case study of Panasqueira Mine in Portugal. *J. Toxicol. Environ. Health A* 74: 1076–86.

Coelho, P., Silva, S., Roma-Torres, J., Costa, C., Henriques, A., Teixeira, J., Gomes, M., and. Mayan, O. 2007. Health impact of living near an abandoned mine—Case study: Jales Mines. *Int. J. Hyg. Environ. Health* 210: 399–402.

Coelho, P., Teixeira, J., and Gonçalves, O. 2011b. Mining activities: Health impacts. In:

Encyclopedia of environmental health, vol. 3, ed. J. O. Nriagu, 788–802. Burlington: Elsevier.

Creason, J., Hinners, T., Bumgarner, J., and Pinkert, C. 1982 Trace elements in hair, as related to exposure in metropolitan New York. *Clin. Chem.* 21: 603–612.

de La Fuente, H., Portales-Perez, D., Baranda, L., Diaz-Barriga, F., Saavedra-Alanís, V., Layseca, E., and González-Amaro, R. 2002. Effect of arsenic, cadmium and lead on the induction of apoptosis of normal human mononuclear cells. *Clin. Exp. Immunol.* 129: 69–77.

Delves, H. T. 1988. Biomedical applications of ICP-MS. *Chem. Britain* 10: 1009–12.

García-Lestón, J., Roma-Torres, J., Vilares, Maria, Rui Pinto, Cunha, L., Prista, João, Teixeira, J. P., Mayan, O., Pásaro, E., Méndez, J., and Laffon, B. 2010. Biomonitoring of a population of Portuguese workers exposed to lead. *Mutat. Res.* 721: 81–88.

Hartwig, A. 1998. Carcinogenicity of metal compounds: Possible role of DNA repair inhibition. *Toxicol. Lett*. 102–103: 235–39.

Hartwig, A., and Schwerdtle, T. 2002. Interactions by carcinogenic metal compounds with DNA repair processes: toxicological implications. *Toxicol. Lett.* 127: 47–54.

He, K. 2011. Trace elements in nails as biomarkers in clinical research. *Eur. J. Clin. Invest*. 41: 98–102.

Heyworth, J. 1990. Evaluation of the Port Pirie Environmental Health Program (Thesis). Adelaide, SA, Australia: Department of Community Medicine, University of Adelaide.

Hernandez-Castro, B., Doniz-Padilla, L.M., Salgado-Bustamante, M., Rocha, D., Ortiz-Perez, M.D., Jimenez-Capdeville, M.E., Portales-Pérez, D.P., Quintanar-Stephano, A., and González-Amaro, R. 2009. Effect of arsenic on regulatory T cells. *J Clin Immunol* 29: 461–69.

Hinwood, A., Sim, M., de Klerk, N., Drummer, O., Gerostamoulos, J., and Bastone, E. 2002. Are 24-hour urine samples and creatinine adjustment required for analysis of inorganic arsenic in urine in urine in population studies? *Environ. Res. Sec. A* 88: 219–24.

Katz, S., and Chatt, A. 1988. *Hair analysis: Applications in the biomedical and environmental sciences*, 133. Weinheim: VCH Verlagsgesellschaft..

Kuangfei, L., Yaling, X., Xuefeng, L., Zouli, W., Bukkens, S., Tommaseo, M., and Paoletti, M. 1999. Metallic elements in hair as a biomarker of human exposure to environmental pollution: A preliminary investigation in Hubei Province. *Crit. Rev. Plant Sci.* 8: 417–28.

Kubota, M., Lin, Y.W., Hamahata, K., Sawada, M., Koishi, S., Hirota, H., and Wakazono, Y. 2000. Cancer chemotherapy and somatic cell mutation. *Mutat. Res.* 470: 93–102.

Ladou, J. 1995. A global ban on lead mining and primary smelting. *Int. J. Occup. Environ.* 1: 70–71.

Lin, T. H., Huang, Y. L., and Wang, M. Y. 1998. Arsenic species in drinking water, hair, fingernails and urine of patients with black-foot disease. *J. Toxicol. Environ. Health A* 53: 85–93.

Lueble, R. W., Chyen, D. H., Dietert, R., Yang, Y., King, M., and Luster, M. I. 2006. The comparative immunotoxicity of five selected compounds following developmental or adult exposure. *J. Toxicol. Environ. Health B* 9: 1–26.

Miekeley, N., Carneiro, M., and Porto da Silveira, C. 1998. How reliable are human hair reference intervals for trace elements? *Sci. Total Environ.* 218: 9–17.

Moszczyński, P., Zabiński, Z., Moszczyński, P. Jr., Rutowski, J., Słowiński, S., and, and Tabarowski, Z. 2001. Immunological findings in cigarette smokers. *Toxicol. Lett.* 118: 121–27.

Nowak, B., and Kozlowski, H. 1998. Heavy metals in human hair and teeth: the correlation with metal concentration in the environment. *Biol. Trace Elem. Res.* 62:213–228.

Orloff, K., Mistry, K., and Metcalf, S. 2009. Biomonitoring for environmental exposures

to arsenic. *J. Toxicol. Environ. Health B* 12: 509–24.

Osugi, Y., Hara, J., Kurahashi, H., Sakata, N., Inoue, M., Yumura-Yagi, K., Kawa-Ha, K., Okada, S., and Tawa, A. 1995. Age-related changes in surface antigens on peripheral lymphocytes of healthy children. *Clin. Exp. Immunol.* 100: 543–48.

Reid, T., Feig, D., and Loeb, L. 1993. Mutagenesis by metal-induced oxygen radicals. *Environ. Health Perspect.* 102: 57–61.

Reis, F., Sampaio, C., Brantes, A., Aniceto, P., Melim, M. Cardoso, L., Gabriel C., Simão, F., and Miguel, J. 2007. Human exposure to heavy metals in the vicinity of Portuguese solid waste incinerators—Part 1: Biomonitoring of Pb, Cd and Hg in blood of the general population. *Int. J. Hyg. Environ. Health* 210: 439–46.

Rodushkin, I., and Axelsson, M. 2000. Application of double focusing sector field ICP-MS multielemental characterization of human hair and nails. Part II. A study of the inhabitants of northen Sweden. *Sci. Total Environ.* 262: 21–36.

Rudy, B. J., Wilson, C. M., Durako, S., Moscicki, A. B., Muenz, L., and, Douglas, S. D. 2002. Peripheral blood lymphocyte subsets in adolescents: A longitudinal analysis from the REACH project. *Clin. Diagn. Lab. Immunol.* 9: 959–65.

Salgueiro, A., Avila, P., Pereira, H., and Silva, E. 2008. Assessment of the impact of pollution by arsenic in the vicinity of Panasqueira Mine (Portugal). *Proceedings of the geoENV 2008— 7th International Conference on Geostatistics for Environmental Applications*, September 8–10, Southampton, UK.

Schrauzer, G., Shrestha, K., and Flores-Arce, M. 1992. Lithium in scalp hair of adults, students, and violent criminals. *Biol. Trace Elem. Res.* 34: 161–76.

Shahabuddin, S., Al-Ayedi, I., Gad El-Rab, M. O., and Qureshi, M. I. 1998. Age-related changes in blood lymphocyte subsets of Saudi Arabian healthy children. *Clin. Diagn. Lab. Immunol.* 5: 632–35.

Schwenk, M., Klein, R., and Templeton, D.2008.Lymphocyte subpopulations in human exposure to metals. *IUPAC Tech. Rep.* 8(6): 1349–64.

Shi, X., Chiu, A., Chen, C.T., Halliwell, B., Castranova, V., and Vallyathan V. 1999. Reduction of chromium (VI) and its relationship to carcinogenesis. *J. Toxicol. Environ. Health B* 2: 87–104.

Slotnick, M., Meliker, J., Avruskin, G., Ghosh, D., and Nriagu, J. 2007. Toenails as a biomarker of inorganic arsenic intake from drinking water and food. *J. Toxicol. Environ. Health A* 70: 148–58.

Soto-Peña, G.A., and Vega, L. 2008. Arsenic interferes with the signaling transduction pathway of T cell receptor activation by increasing basal and induced phosphorylation of Lck and Fyn in spleen cells. *Toxicol. Appl. Pharmacol.*. 230: 216–26.

Stephens, C., and Ahern, M. 2001. *Worker and community health impacts related to mining operations internationally. A rapid review of the literature*. Paper prepared for MMSD. London: IIED and WBCSD.

Tanigawa, T., Araki, S., Sata, F., Nakata, A., and Araki, T. 1998. Effects of smoking, aromatic amines, and chromates on CD4 and CD8 T lymphocytes in male workers. *Environ.Res.* 78: 59–63.

Taooka, Y., Takeichi, N., Noso, Y., Kawano, N, Apsalikov, K., and Hoshi, M. 2006. Increased T-cell receptor mutation frequency in radiation-exposed residents living near the Semipalatinsk nuclear test site. *J. Radiat. Res.* 47: 179–181.

Taylor, A., Branch, S., Day, M., Patriarca, M., and White, M. 2011. Atomic spectrometry update: Clinical and biological materials, foods and beverages. *J. Anal. Atom. Spectrom.* 26: 653–92.

U.S. Environmental Protection Agency. 1992. *Record of decision—Bunker Hill Mining and Metallurgical Complex, Soshone County, Idaho*. Available at http://www.epa.gov/superfund/sites/rods/fulltext/r1092041.pdf

Valkovic, V. 1988. *Trace elements in human hair*, vol. 2. Boca Raton, FL: CRC Press.

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Abstract

Human health is constantly affected by a multitude of agents present in the surrounding environment. Biomarkers are key factors, both in clinical health risk assessment and environmental risk assessment. Traditionally, they are classified in three different classes: exposure, effect, and susceptibility. Their main usefulness relies on their ability to link hazard exposure to the pathogenesis of human disease. The development and validation of biomarkers as early predictors of clinical disease is a top priority, improving health risk assessment and contribute to new disease prevention policies in environmental and occupational settings.

Keywords

Biological monitoring, Biomarkers, Environmental exposure, Environmental monitoring, Genetic susceptibility, Human health, Internal dose, Molecular epidemiology, Mutation assays, Occupational exposure, Risk assessment

Biographical Sketch

Patrícia Coelho's current research is focused on the health effects of environmental and occupational exposure to metal(loid)s contamination resulting from mining activities, using a multistage approach, in order to integrate different biomarkers. Studied biomarkers include biomarkers of exposure (internal dose: chemical and/or metabolites in urine, blood, nails, and hair), biomarkers of effect (cytogenetics: micronucleus, chromosomal aberrations, and sister chromatid exchange; and DNA damage: comet assay), and biomarkers of susceptibility (genetic polymorphisms: genes involved in xenobiotic metabolism and DNA repair). She also collaborate in several research projects in human biomonitoring field.

João Paulo Teixeira has been working, in the last 15 years, in the field of environmental health, mainly in genetic damage caused by genotoxic and carcinogenic compounds. The effects of genotoxic xenobiotics are studied in a multistage approach, including environmental monitoring (external exposure), biomarkers of exposure (chemical and/or metabolites in urine and blood,), biomarkers of effect (micronucleus in lymphocytes and buccal cells, sister chromatid exchange, chromosomal aberrations, and comet assay), and biomarkers of susceptibility (polymorphisms of genes involved in xenobiotic metabolism and DNA repair). He is focused on the molecular mechanisms involved in the cascade of genotoxic/carcinogenic events in relation to exposure to xenobiotics, as well as on the factors of individual susceptibility modulating genotoxic and/or carcinogenic effects. He is the Principal Investigator of two funded national projects in the field of Environmental Health and is the Portuguese coordinator of a European project in the field of nanotoxicology – 'NanoLINEN – Nanotoxicology link between India and European Nations' – European Project Networking Pilot Programme on Biotechnology and Health of New INDIGO.

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a0005

Biomarkers, Human Health

AU1

PCDS Coelho and JPF Teixeira Environmental Health Department, Portuguese National Institute of Health, Porto, Portugal

s0010 ## Introduction

p0010 In order to better protect the human health, a process of population study known as molecular epidemiology has been developed to integrate laboratory measurements with epidemiological methodologies, linking individual exposure to an important biological event. The biological component is evaluated with biological markers, also called biomarkers. Unlike their usage for many years in the medical field, they have been used only for 3 decades in the environmental health field.

p0015 In 2001, the World Health Organization defined biomarker as a chemical, its metabolite, or the product of an interaction between a chemical and some target molecule or cell that is measured in the human body. They commonly include biochemical, molecular, genetic, immunologic, or physiologic signals of events in biologic systems. The events are represented as a continuum between an external exposure to an agent and the resulting clinical effects (Figure 1). Exposure to these agents can happen through contact with contaminated air, water, soil, and food and also in the occupational environment and lifestyle factors. All these routes contribute to a complex exposure situation in daily life.

p0020 Along with the concept of biomarker, comes the concept of biological monitoring or biomonitoring. In 2011, the Centers for Disease Control and Prevention defined human biomonitoring as the direct measurement of people's exposure to toxic substances in the environment by measuring the substances or their metabolites in human specimens, such as blood or urine. In other words, the assessment of human exposure via the measurement of biomarkers. Concentrations found can then be related to the internal dose and consequently investigate the possible association between these data toward the effect or back to possible source of exposure. Health risk assessment should include the measurement of actual impacts on biological endpoints from contamination in soil, surface water, groundwater, air, and sediments at various levels of biological organization.

p0025 The measurement of biomarkers in population studies requires the appropriate study design. This includes the choice of the appropriate matrix, adequate collection, shipping, and storage and also the implementation of analytical techniques that fulfill the requirements to properly execute the study.

p0030 In the last decade, great advances have been achieved in the biomarker field, but there are still some issues/limitations that need to be taken into account in future investigations.

s0015 ## Classes of Biomarkers

p0035 There are different classes of biomarkers. Traditionally, these are classified as biomarkers of exposure, effect, and susceptibility (Figure 1). Each of which is used to answer different questions and their accurate interpretation depends on the knowledge of the various transformations occurring in the metabolic pathways of the human body.

p0040 In 2006, the Committee on Human Biomonitoring for Environmental Toxicants of the National Research Council as defined the three categories as:

u0010 - Biomarkers of exposure: a chemical, its metabolite, or the product of an interaction between a chemical and some target molecule or cell that is measured in the human body.
u0015 - Biomarkers of effect: a measurable biochemical, physiologic, behavioral, or other alteration in an organism that, depending on the magnitude, can be recognize as associated with an established or possible health impairment or disease.
u0020 - Biomarker of susceptibility: an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific chemical substance.
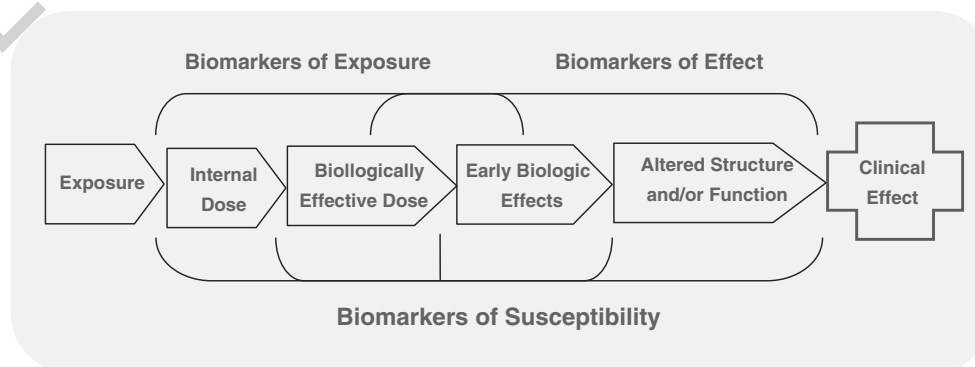
AU2 f0010 **Figure 1** Simplified diagram of the three categories of biomarkers along with the biologic events taking place between exposure and clinical effect.

t0010 **Table 1**   Examples of the most used biomarkers in each (sub)category. Based on the published literature review performed by Au et al. (2007)

AU3

Biomarkers of exposure		Biomarkers of effect	
Internal dose	*Biologically effective dose*	*Early biologic effect*	*Altered structure and/or function*
Parent compound/metabolites	DNA/protein adducts		
		Reporter gene mutation	
		Altered gene expression	
		DNA strand breaks	
		Micronuclei	
			Chromosomal aberrations
			Cancer gene mutation
	Biomarkers of susceptibility		
	DNA sequence variations		

p0060   In order to achieve the mechanistic understanding of the biologic effect, and therefore a better prediction of disease risk, appropriate biomarkers of each category should be used. Examples of the most commonly used biomarkers are presented in Table 1.

s0020 Biomarkers of Exposure

p0065 Biomarkers of exposure can be divided in two subcategories: internal dose and biologically effective dose.

p0070   Biomarkers of internal dose aim to determine the compound or its metabolites in tissues or body fluids such as blood, urine, breast milk, and saliva. They can also give information on other sources of exposure to that compound and the existence of genetic polymorphisms for metabolic enzymes. Biomarkers of biologically effective dose assess the interaction of compounds with molecular targets such as DNA and protein receptors (e.g., measurement of DNA and protein adducts in urine and serum). Despite the presence of these adducts being readily measured, DNA adducts have become more popular and one of the most important biomarkers of exposure as their presence may be indicative of the risk associated with the exposure.

p0075   Although biomarkers of exposure are highly relevant and specific indicators of an exposure, the information given does not necessarily translate into prediction of health consequence, and therefore other biomarkers need to be analyzed.

s0025 Biomarkers of Effect

p0080 Biomarkers of effect can be divided in two subcategories: early biological effects and altered structure and/or function.

p0085   Biomarkers of early biological effects have improved accuracy for exposure assessment, providing objective measures on potential health effects at the level of the individual. They include several markers such as reporter gene mutation (e.g., *HPRT*, *HLA*, *GPA*, and *TCR* gene mutation assays), altered gene expression (e.g., expression from metabolizing genes, DNA repair gene, and specific enzymes), DNA strand breaks (quantified by the comet assay), cytogenetic markers such as micronuclei and chromosomal aberration (CA). From all the early biological effect markers, CA assay is the most widely used and best validated biomarker. The mechanisms are better understood and most environmental toxic substances have been shown to induce them. As CAs are also markers of altered structure and/or function, they are extremely useful in cancer risk assessment. Most cancer cells and developmental abnormalities present these alterations. Along with CAs, cancer gene mutation such as tumor suppressor genes and oncogenes are predictive markers for cancer morbidity and mortality.

Biomarkers of Susceptibility   s0030

The expression of all the previous described biomarkers is p0090 significantly influenced by individual factors, acquired (e.g., life styles like smoking habits and alcohol consumption), and genetic susceptibility categories.

It is well known and recognized that even under identical p0095 exposure conditions, different individuals have different responses. Therefore, some individuals are more susceptible/resistant to the exposure than others. In order to identify these variations, investigations with biomarkers of susceptibility have focused on DNA sequence variation in certain genes, such as the ones involved in chemical metabolism and DNA repair, genes related to immune function, and cell cycle control. These studies provide valuable information about the influence of such genes on specific effects of exposure(s) and response to genotoxic agents.

Usefulness vs. Limitations   s0035

Biomarkers are key factors in human health risk assessment, p0100 both in clinical and environmental field – environmental risk assessment (ERA), providing reliable and specific information on the etiology and mechanisms of disease process, thus for disease prevention. Their usefulness is based on the ability to measure integrated exposures via all routes without being susceptible to complex and extensive assumptions or models (biological monitoring vs. environmental monitoring – Figure 2). In environmental monitoring, exposure models are built and usually involve applying sets of standardized assumptions about activity levels, dietary choices, behavior, routes of exposure, routes of absorption, and many other factors. There is always both uncertainty and error attached to these estimations. In biological monitoring, the main concept is the measurement of the environmental contaminant in the body (internal dose). This is the amount absorbed via all

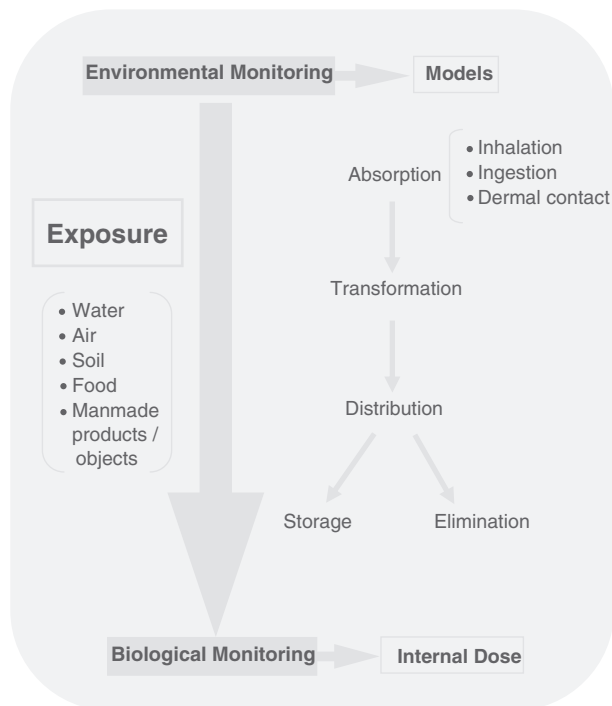f0015 **Figure 2** Sources of exposure and main pathways in environmental and biological monitoring.

Table 2 Main advantages and limitations of biomarkers   t0015

Advantages	Limitations
Confirm the actual amount absorbed via all routes	Many requirements to fulfill in all the process
Help to test and validate exposure models	Only few are properly validated
Detect contaminants at trace levels	Difficult to define the toxic dose
Create individual exposure trends	Sources or pathways of exposure are not defined
Verify the effectiveness of public health interventions	Reference ranges need to be established

are crucial for meaningful results. Numerous biomarkers are only used for research purpose and not clinical at the individual patient level. This is due to the fact that many lack the precision necessary for clinical utility and are often highly sensitive to poor laboratory technique.

The most common matrixes used in biomarkers research are blood, urine, expelled air, and breast milk. Other matrixes such as nails and hair have been increasingly used in recent studies as they are noninvasive and relatively easy to collect. Additionally, they can be completely self-administered, stored at room temperature, and no specific equipment is needed. Nevertheless, problems with contamination, lack of standardized procedures for collection, washing, and digestion, and with the significance of obtained results need to be solved in this field in order to validate such matrixes as reliable biomarkers.   p0125

Until now, there are no reference ranges or safe ranges that describe general population exposures to contaminants for the majority of biomarkers. Also, a lack of toxicological and epidemiological information makes it often impossible to know if the exposures measured by biomarkers are below or above the toxic limit. Unless there are some studies in this field that defined the toxicity and the dose response curve for the specific contaminant, the results obtained are rather difficult to interpret.   p0130

Another important factor is that each biomarker has its own characteristics relating to sensitivity, specificity, and toxicity. Expression of some may not be highly relevant in the carcinogenic process as they represent tolerable and most possible reversible biological changes (e.g., lipid peroxidation and sister chromatid exchange). Also, most times one cannot define the toxic dose nor define sources or pathways of exposure being difficult to interpret obtained results. Choosing the appropriate biomarkers that indicate exposure to the specific agent under study and that represent biological events along the pathway from exposure to clinical effect leads to a mechanistic understanding of biological effects and consequent better prediction of disease risk.   p0135

Ethical issues are also one of the most important subjects when dealing with biological sampling. Before collecting the biological specimens, the goals of the study must be well explained, and an informed consent needs to be signed. Research protocols demand that samples have to be anonymized by coding them. National and international regulations need to be follow when handling genetic material and banking it for long time. Communication of results to studied   p0140

routes – ingestion, inhalation, and dermal – moving through the metabolic pathways. It is transformed and either stored or eliminated. Biomarkers can help to test and validate exposure models by comparing the results obtained in environmental monitoring (exposure models) to the ones obtained in the biological monitoring (internal dose).

p0105 In the last decades, analytical techniques have become extremely sensitive and accurate allowing the detection of exceptionally low-level exposures. This is of enormous importance as most biological matrixes are complex, some of them only available in small volumes, and the majority of chemicals are present only at trace levels.

p0110 Other uses of biomarkers are related to the possibility of creating individual exposure histories when it is measured in an individual over a period of time and also the ability to evaluate the effectiveness of public health interventions like withdraw, restriction, or reformulations of chemicals. Continuous biomonitoring can determine if exposure to those chemicals actually decreased over the time period being studied.

p0115 Despite their enormous advantages, there are several issues/limitations (Table 2).

p0120 One of the most important is the construction of an appropriate study design for the investigation before selecting which biomarkers should be used. To provide the linkage between exposure and disease, they must be measured in the correct matrix, have an adequate sample size and combination of appropriate biomarkers, using the most reliable and reproducible analytical technology available. Accordingly, highly sensitive, specific, and selective multianalytical methods for the extraction, separation, and quantification must be developed. Also standardized collection, storage, and processing conditions

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individuals is also a crucial subject. Only medical and/or scientific experts should have access to them and then choose the most appropriate way to analyze, interpret, and communicate them.

Future Perspectives/New Fields of Application

p0145 Nowadays, a top priority in biomarkers research is the development and validation of biomarkers linking environmental exposures to the etiology of human disease. They can then be widely used as biomarkers as early predictors of clinical disease enhancing health risk assessment and contributing to effective new tools for human risk assessment. Well-designed studies including clear identification of the assessment objective, rational selection of biomarkers with proper consideration of specific pathways related to specific exposures to assess individual susceptibility, and development of strategies for the evaluation of the impact of complex genetic variations on the overall pathway efficiency with reliable and efficient phenotypic assays need to be performed. Along with the validation, it is also important to perform the qualification of used biomarkers. For analytical measurements, interlaboratory comparison trials should be made. Improved modeling methods for interpreting biomonitoring data using calibrate and validate models such as pharmacokinetics are also needed.

p0150 Recently, the Council of the European Union emphasized the need to consider combined and mixed exposures of chemicals in future risk assessments. Actions like prioritizing chemicals of major concern, evaluating if there are enough tools to obtain information on internal dose biomarker, and then model it both in a forward and reverse direction must be carried out.

p0155 It is also important to perform research activities including characterization of a baseline for biomarkers, application of statistical methods to assess temporal departures from that baseline, and establishment of a database of biomarker disease associations, with null and negative studies, resulting in an advance in scientific understanding and application of biomonitoring data.

p0160 Another major challenge is to establish reference ranges for the majority of biomarkers. A large amount of studies need to be conducted as mixed and often conflicting results have been obtained in studies of the distribution of biomarkers in populations environmentally and/or occupationally exposed to hazardous agents. This will allow for the development of legislation, methodology, and to support research within this area.

p0165 A vast amount of research data comes from clinical trials. Biomarkers used in this field require a robust linkage or correlation with clinical endpoints. A cooperation between those working in the clinical field and in the ERA will be crucial to implement appropriate programs for health risk assessment and contribute to valuable new disease prevention policies in environmental and occupational settings.

p0170 New information available from genome-derived methods is of paramount importance. Building reliable biomarker databases will lead to integration of information from the genome programs to expand the scientific borders on etiology, health risk prediction, and prevention of environmental disease.

p0175 Further studies should also include the application of more recent technologies such as gene arrays, proteomics, and protein activity profiling. These techniques allow the monitoring of a large number of genes and the analysis of alterations in gene expression after combined exposure. This will help to develop potential new biomarkers as screening tools to identify improved candidates for biomonitoring. A combination of approaches at different levels of cellular organization, such as DNA, RNA, and proteins, could be the optimal biomarker.

p0180 Other challenges are to establish the link between exposure and health effects using a biological systems approach, changing toxicity test from using animal tests to cell-based techniques. The main goal of this field is to obtain irrefutable data showing that pathway perturbations can predict results from animal testing. Until now, no consensus was obtained on which type of cell should be used.

p0185 In the last 2 decades, an explosion in information and literature on human studies with different classes of biomarkers has occurred. Globally, one can see promising future directions in biomarkers research with several new fields of investigation and new technologies being applied.

See also: 00451; 00553; 01000.

Further Reading

Albertini, R., Bird, M., Doerrer, N., Needham, L., Robinson, S., Sheldon, L., Zenick, H., 2006. The use of biomonitoring data in exposure and human health risk assessment. Environ. Health Perspect. 114, 1755–1762.

Au, W.W., 2007. Usefulness of biomarkers in population studies: from exposure to susceptibility and to prediction of cancer. Int. J. Hyg. Environ. Health 210, 239–246.

Bonassi, S., Au, W., 2002. Biomarkers in molecular epidemiology studies for health risk prediction. Mutat. Res. 511, 73–86.

Bonassi, S., Neri, M., Puntoni, R., 2001. Validation of biomarkers as early predictors of disease. Mutat. Res. 480–481, 349–358.

Farmer, P., Kyrtopoulos, S., Emeny, J., 2007. State of Validation of Biomarkers of Carcinogen and Early Effects and Their Applicability to Molecular Epidemiology. Nofer Institute of Occupational Medicine, Poland.

Gil, F., Pla, A., 2001. Biomarkers as biological indicators of xenobiotic exposure. J. Appl. Toxicol. 21, 245–255.

Kyrtopoulos, S.A., 2006. Biomarkers in environmental carcinogenesis research: striving for a new momentum. Toxicol. Lett. 162, 3–15.

Needham, L.L., Calafat, A.M., Barr, D.B., 2007. Uses and issues of biomonitoring. Int. J. Hyg. Environ. Health 210, 229–238.

Owen, R., Galloway, T.S., Hagger, J.A., Jones, M.B., Depledge, M.H., 2008. Biomarkers and environmental risk assessment: guiding principles from the human health field. Mar. Pollut. Bull. 56, 613–619.

Silins, I., Hohberg, J., 2011. Combined toxic exposures and human health: biomarkers of exposure and effect. Int. J. Environ. Res. Public Health 8, 629–647.

Mining Activities: Health Impacts

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Abbreviations

AMD	acid mining drainage
ARD	acid rock drainage
ATSDR	Agency for Toxic Substances and Disease Registry
CERCLA	Comprehensive Environmental Response, Compensation and Liability Act
EIS	Environmental Impact Statement
EU	European Union
HIV/ AIDS	human immunodeficiency virus/acquired immunodeficiency syndrome
IARC	International Agency for Research on Cancer
NEPA	National Environmental Policy Act
NGO	nongovernmental organization
OSH	occupational safety and health
RCRA	Resource Conservation and Recovery Act
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

Glossary

Adit   a horizontal or nearly horizontal passage driven from the surface for the working or dewatering of a mine.

Alluvial   said of a placer formed by the action of running water, as in a stream channel or alluvial fan; also said of the valuable mineral, for example, gold or diamond, associated with an alluvial placer.

Asbestos   a commercial term applied to silicate minerals that separate readily into thin, strong fibers that are flexible, heat resistant, and chemically inert, thus making them suitable for uses (as in yarn, cloth, paper, paint, brake linings, tiles, insulation, cement, fillers, and filters) where incombustible, nonconducting, or chemically resistant material is required.

Asbestosis   a lung disease caused by breathing asbestos dust.

Biomarker   a term expressing specific measurements of an interaction of a biological system with a toxic agent. Biomarkers can be divided into biomarkers of exposure, effect, and susceptibility. Biomarkers of exposure may be the xenobiotic chemical itself or its metabolites (internal dose) or the product of an interaction between the chemical and its target biomolecule (biologically effective dose). Biomarkers of effect may be an endogenous component, a measure of the functional capacity, or some other indicator of the organs, system, or body condition that might be affected by the exposure. Biomarkers of susceptibility indicate that the individual is particularly sensitive to the effect of a xenobiotic or to the effects of a group of such compounds.

Boulder   a detached rock mass larger than a cobble, having a diameter greater than 10 in (25.4 cm) or 8 phi units, or about the size of a volleyball, being somewhat rounded or otherwise distinctively shaped by abrasion in the course of transport; the largest rock fragment recognized by sedimentologists.

Carbon leach   the waste product remaining after the desired metals have been removed from activated carbon.

Crud   a solid-stabilized emulsion that tends to collect at the aqueous/organic interface in the settler of a solvent extraction circuit.

Drainage   the manner of gravity flow of water or the process of channelization, for removal at a point remote from a mining operation.

Dredging   removing solid matter from the bottom of an area covered by water.

Dross   the scum that forms on the surface of molten metals largely because of oxidation, but sometimes because of the rising of impurities to the surface, and which contains metal and metal oxides.

Electrometallurgical operation   the use of electric and electrolytic processes to purify metals or reduce metallic compounds to metals.

Electrowinning slim   a material of extremely fine particle size produced in an electrochemical process in which a metal dissolved within an electrolyte is plated onto an electrode.

Emphysema   a chronic (long-term) lung disease that can get worse over time. It is usually caused by smoking. Having emphysema means some of the air sacs in lungs are damaged, making it hard to breathe.

Epidemiology   a public health discipline combining statistical and medical investigation methods to study the distribution and the determinants of health-related states and events in populations. The ultimate purpose of epidemiology is to improve the public health by contributing to the prevention, mitigation, or treatment of health problems.

Flotation water   water produced during milling process in which valuable mineral particles are induced to become attached to bubbles and float as others sink.

Heap leaching   a process used for the recovery of copper, uranium, and precious metals from weathered low-grade ore. The crushed material is laid on a slightly sloping, impervious pad, and uniformly leached by the percolation of the leach liquor trickling through the beds by gravity to ponds. The metals are recovered by conventional methods from the solution.

Highwall   the unexcavated face of exposed overburden and coal or ore in an open cast mine or the face or bank on the uphill side of a contour strip mine excavation.

Hydrometallurgical operation   it involves the use of liquid reagents in the treatment or reduction of ores.

Leaching   dissolution from ore or concentrates after suitable comminution to expose the valuable minerals, by aqueous and chemical attack. If heat and pressure are used to intensify or speed this, the work is called pressure leaching.

Leucopenia   an abnormal lowering of the white blood cell count.

Limestone   a rock consisting chiefly of calcium carbonate or carbonate of lime.

Lime   oxide of calcium – CaO; the white or gray, caustic substance, usually called quicklime, obtained by calcining limestone or shells, the heat driving off carbon dioxide and leaving lime.

Mesothelioma   a rare form of cancer in which malignant (cancerous) cells are found in the mesothelium, a protective sac that covers most of the body's internal organs.

Mill   a mineral treatment plant in which crushing, wet grinding, and further treatment of ore is conducted. Also, separate components, such as ball mill, hammer mill, and rod mill.

Outcrop   the part of a rock formation that appears above the surface of the surrounding land.

Overburden   designates material of any nature, consolidated or unconsolidated, which overlies a deposit of useful materials, ores, or coal – especially those deposits that are mined from the surface by open cuts.

Particulate matter   the generic term used for a type of air pollution that consists of complex and varying mixtures of particles suspended in the air, which are breathed.

Pit   a mine, quarry, or excavation worked by the open-cut method to obtain material of value.

Pneumoconiosis   the deposition of particulate matter such as asbestos and silicon in the lungs.

Potliner   a specialized form of electrolytic cell liner used in the aluminum production process. Potliners may contain toxic levels of arsenic and selenium, as well as detectable levels of cadmium, chromium, barium, lead, mercury, silver, sulfates, and cyanide.

Pyrometallurgical operation   it consists of the thermal treatment of minerals and metallurgical ores and concentrates to bring about physical and chemical transformations in the materials to enable recovery of valuable metals. Pyrometallurgical treatment may produce saleable products such as pure metals, or intermediate compounds or alloys, suitable as feed for further processing.

Quarry   an open or surface mineral working, usually for the extraction of building stone, as slate, limestone, and so on. It is distinguished from a mine because a quarry is usually open at the top and front, and, in ordinary use of the term, by the character of the material extracted.

Radionuclide   an atom with an unstable nucleus, characterized by excess energy, which is available to be imparted either to a newly created radiation particle within the nucleus or else to an atomic electron. The radionuclide, in this process, undergoes radioactive decay, and emits gamma ray(s) and subatomic particles. These particles constitute ionizing radiation. Radionuclides may occur naturally, but can also be artificially produced. They are often referred to as radioactive isotopes or radioisotopes, and play an important part in the technologies that provide with food, water, and good health. However, they can also constitute real or perceived dangers.

Refining   the purification of crude metallic products.

Roaster fines   a reverberatory furnace or a muffle used in roasting ore.

Roasting   heating an ore to affect some chemical change that will facilitate smelting.

Seepage   the slow movement of water through the pore spaces of a solid material. This term is also applied to a loss of water by infiltration through the bottom of a stream, canal, irrigation ditch, reservoir, or other body of water.

Shaft   an excavation of limited area compared with its depth; made for finding or mining ore or coal, raising water, ore, rock, or coal, hoisting and lowering workers and material, or ventilating underground workings. The term is often specifically applied to an approximate vertical shaft, as distinguished from an incline or inclined shaft. A shaft is provided with a hoisting engine at the top for handling workers, rock, and supplies; or it may be used only in connection with pumping or ventilating operations.

Silicosis   a form of lung disease resulting from occupational exposure to silica dust over a period of years. Silicosis causes slowly progressive fibrosis of the lungs, impairment of lung function, and a tendency to tuberculosis of the lungs.

Siltation   the deposition or accumulation of silt that is suspended throughout a body of standing water or in

some considerable portion of it; especially the choking, filling, or covering with stream-deposited silt behind a dam or other place of retarded flow, or in a reservoir. The term often includes sedimentary particles ranging in size from colloidal clay to sand.

Sinking injection   extending excavations downward at or near the vertical plane series of wells are drilled into the ore body and a solvent circulated through the formation by injection.

Slag   the waste product of the process of smelting.

Slope   primary inclined opening, connecting the surface with the underground workings.

Slurry   the fine carbonaceous discharge from a mine washery. All washeries produce some slurry, which must be treated to separate the solids from the water to have a clear effluent for reuse or discharge.

Smelting   the chemical reduction of a metal from its ore by a process usually involving fusion, so that earthy and other impurities separate as lighter and more fusible slags and can readily be removed from the reduced metal. An example is the reduction of iron ore (iron oxide) by coke in a blast furnace to produce pig iron. Smelting may also involve preliminary treatment of ore, such as by calcination and further refining processes, before the metal is fit for a particular industrial use.

Spent furnace (refractory) brick   this material, as its name implies, is from the furnace or refractory liner and is generated in a relatively small quantity.

Spent leach   the waste product remaining after the leach operations.

Subsidence   the sudden sinking or gradual downward settling of the earth's surface with little or no horizontal motion. The movement is not restricted in rate, magnitude, or area involved. Subsidence may be caused by natural geological processes, such as solution, thawing, compaction, slow crustal warping, or withdrawal of fluid lava from beneath a solid crust; or by human activity, such as subsurface mining or the pumping of oil or groundwater; bottom subsidence.

Tailing   the gangue and other refuse material resulting from the washing, concentration, or treatment of ground ore.

Tailing impoundment   a reservoir for impounding. Used in connection with the storage of tailings from ore-dressing plants and hydraulic mines.

Tailing pound   area closed at lower end by constraining wall or dam to which mill effluents are run. Clear water may be returned after settlement in dam, via penstock(s) and piping.

Void   a general term for pore space or other openings in rock. In addition to pore space, the term includes vesicles, solution cavities, or any primary or secondary openings.

Well   a borehole or shaft sunk into the ground for the following purposes: obtaining water, oil, gas, or mineral solutions from an underground source; introducing water, gas, or chemical reagent solutions under pressure into an underground formation; or removing the leachate from such an operation.

Introduction

The earth has many natural resources on which people depend that must be mined. Mining is one of the oldest and most important activities in the history of human civilization as it provides the raw ingredients to most of the materials available. Mining is essential for nations to have adequate and steady supplies of minerals to fulfill their economic and defense needs at suitable environmental, energy, and economic costs.

The use of minerals by countries worldwide is extensive and includes electrical generation, production of cement, steel, agricultural lime, commercial and residential building materials, asphalt, and medicines, as well as countless household, electronic, and other manufactured products.

Mining industry is a vital economic sector for many countries but is also one of the most hazardous activities in occupational and environmental context.

Mining Operations

The definition of a mine site may be wide. In 1982, the U.S. Environmental Protection Agency (U.S. EPA) has defined a mine as "an area of land upon, or under, which minerals or metal ores are extracted from natural deposits in the earth by any methods, including the total area upon which such activities occur or disturb the natural land surface."

There are several methods used to mine a specific mineral commodity. Mineral commodities can be grouped into three broad categories: metallic minerals, nonmetallic or industrial minerals, and fuel minerals. Examples of minerals belonging to these three groups are shown in **Table 1**.

Table 1   The three main groups of mineral commodities

Mineral commodity	Examples
Metallic	*Precious metals* – gold, silver, platinum *Base metals* – copper, lead, iron, zinc, nickel
Nonmetallic	Asbestos, gypsum, limestone, sand, salt gravel
Fuels	Coal, peat, natural gas, petroleum, uranium

Some of the minerals produced such as coal and salt are ready to be used right after they have been mined. It may be necessary to wash or treat these commodities in different ways to increase their quality, but their properties remain almost unchanged.

Metals and some of the nonmetals usually occur in nature as ores and so they must be treated. An ore is a rock containing a sufficiently high concentration of useful minerals or metals to justify commercial exploitation, and also materials without commercial value called gangue.

Mining operations consist essentially of four steps: exploration, mining, mineral processing and dressing, and metallurgic processing.

Exploration is an activity generally performed before mining and conducted to locate deposits of economic interest. Since the 90s, exploration has made an increasing use of satellite images, but traditional methods such as deep drilling and searching for surface outcrops are still broadly used. Among other activities are the preparation of access routes, topographic and geological mapping, establishment of camps and auxiliary facilities, geophysical works, hydrogeological research, opening up of reconnaissance trenches and pits, and sampling.

Mining involves removal of the desired minerals from the surface. Mining processes can be divided into surface mining, underground mining, and *in situ* mining. The first one is used to excavate ores at or close to the earth's surface and it includes open pit mining (also called highwall or strip mining) and dredging. Open pit mining involves excavation of an area of overburden and removal of the ore exposed in the resulting pit. Dredging is used to excavate placer deposits, which are concentrations of heavy metallic minerals that occur in sedimentary deposits associated with current or ancient watercourses. Underground mining removes mineral by extracting under the surface and removing the ore. Finally, *in situ* mining removes minerals by sinking injection and extraction wells, and leaching the ore in place.

Main wastes produced in this stage can be grouped in overburden, mine water, and waste rock. The former is generated at surface mines to expose the ore beneath (i.e., topsoil and rock), and it should be stored and stockpiled for use in reclamation during closure or decommissioning. Sources of mine water are groundwater seepage, surface water inflow, or direct precipitation. These waters may contain significant concentrations of heavy metals and altered pH (mainly highly acidic). Waste rock consists of nonmineralized and low-grade mineralized rock removed from, around, or within the orebody during extractions activities. It includes granular, broken rock, and soils ranging in size from fine sand to large boulders. Geochemistry of waste rock varies widely with mine type and over time as differing lithologic strata is exposed.

Next step is mineral processing and dressing that refers to all the mechanical, physical, and chemical methods used to separate minerals from the gangue and to partially treat them. This is generally the most polluting stage and the one that generates the highest number of health risks. It includes three types of operations: preparation, concentration, and conditioning. At the end of these three stages, the ore will be more concentrated but the purity achieved is relatively low.

Some of the wastes produced in these operations are known as tailings (Figure 1).

The physical and chemical nature of tailings varies with the ore being milled and the milling operations used. There are three types of tailings depending on water content: wet, thickened, and dry. Tailings may be a source of contamination, even though they have much lower concentrations of the target mineral(s) than the mined ore. This is due to the presence of sulfides such as pyrite, metals, and reagents added during preceding step. Tailings can be managed in tailing impoundments (wet or thickened) or disposable piles (dry). Certain tailings may be slurried as backfill into underground mines or disposed in underwater.

There are other types of wastes produced in these operations. Several types of leaching wastes and hazardous material, such as dump and heap leach waste, spent leachate and spent carbon, electrowinning slimes, and crud are formed. If these wastes are not correctly managed, they can be potential sources of contamination of groundwater, surface water, and soil when transported by windblown dust or stormwater erosion. Acid drainage may be generated from the oxidation of sulfide ores and must be controlled.

The final step is metallurgical processing. It refers to melting and refining operations that are used to produce

Figure 1   Picture of a tailing originated from wastes of mining activities.

pure metals or to prepare alloys. Part of these processes can be carried out on site, but they usually occur someplace else. This process includes several operations, namely, pyrometallurgical operations (e.g., smelting, refining, and roasting), hydrometallurgic operations (e.g., digestion of phosphate in producing phosphoric acid), and electrometallurgic operations (e.g., electrolytic refining).

Similar to the other processes this one also produces different types of mineral processing wastes, such as slag and dross, spent furnace (refractory) brick, potliner, and roaster fines. Several of these wastes are recycled back to the mineral processing facilities as they contain high levels of metals but others are disposed or dispersed at the mine site contaminating the environment with heavy metals, sulfates, cyanides, and many other substances. The production of large amounts of waste material (often very acidic) and particulate emissions has led to major environmental and health concerns.

It is crucial to manage wastes produced in each operation to significantly reduce environmental impact of mining activities.

Mine Closure

Minerals are a nonrenewable source and therefore the life of mines is finite. The closure of a mine may also be due to low value of the commodity, making mining unprofitable, technical difficulties, and national security.

The mining life cycle (Figure 2) can be divided into some steps ending with mine closure.

Closure normally means cessation of all mining at a site. As some temporary disruption to the environment is unavoidable when mining, mine closure generally requires some environmental activities such as a removal of hazards, recontouring, and planting where possible.

It involves completing a reclamation plan that ensures the safety of areas affected by the mining operations. The Surface Mining and Control Act of 1977 states that "reclamation must restore the land affected to a condition capable of supporting the uses which it was capable of supporting before any mining, or higher or better uses."

Closure happens when all programmed reclamation plans have been concluded and safety of the area is guaranteed. Protecting the environment through reclamation is an essential part of mining. It is possible for mining companies using modern mining technology to return mine land as close as possible to the way it was before mining occur. Reshaping the surface stabilizes slopes and drainages, waste rock piles, tailing ponds, highwalls, and access roads. Therefore, mining can be executed with concern for health and respect for ecological requests.

The restoration of stream channels allows the reintroduction of plants and animals that were gone because of mining. Cleanup and treatment of hazardous materials avoids additional harm of the environment. Usually small amounts of mining-related materials, such
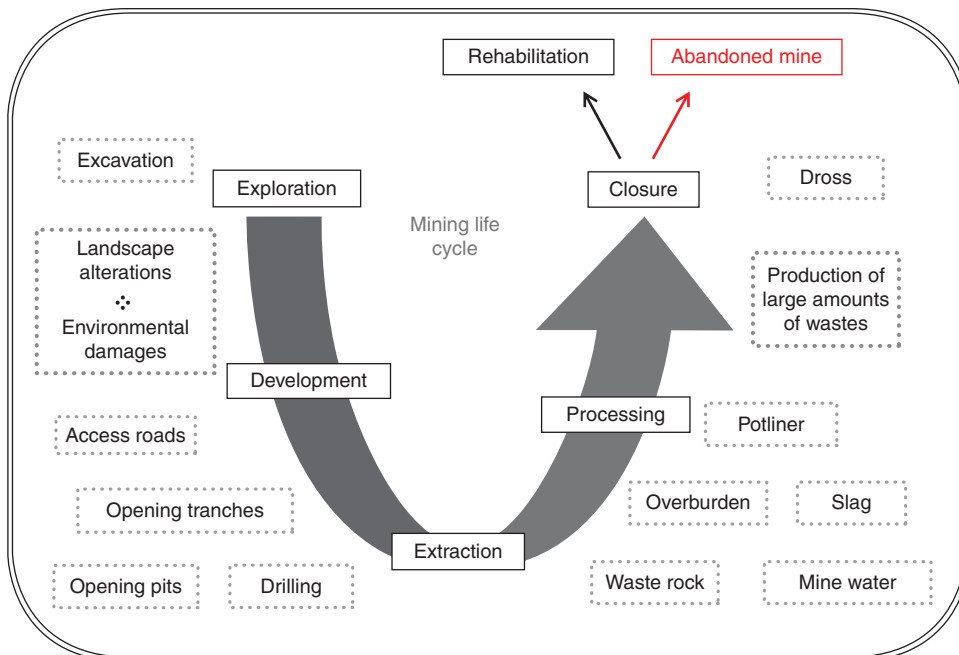
Figure 2   Schematic representation of the main steps taking place in mining life cycle with the consequent impacts in the environment. Also represented are the several wastes produced in each step.

as chemicals or fuels are fully removed, but large quantities of naturally occurring materials, such as unweathered waste rock that produces acids, may be treated on site through the application of lime, which will provide a buffer. In more extreme cases, limestone drains or artificial wetlands can be used to filter heavy metals and decrease acidity. Revegetation of mine sites allows restoration of native plant population and patterns, leading to natural succession. Time and nature then restore the natural productivity in the site.

Mine closure legislation should obligate authorities to control and prevent mines to become abandoned by setting up funds to rehabilitation programs.

Abandoned Mines

Abandoned mines are a very important global concern as they pose real or potential threats to human safety and health, as well as environmental damages. Abandoned or inactive mines are generally sites where highly developed exploration, mining, or mine production ceased without rehabilitation.

Rehabilitation programs usually require huge fund amounts. Therefore, several issues such as who is responsible to provide the funds and to do the rehabilitation work, what mechanisms exist in various jurisdictions to raise the funds are raised. When no owners are identified or when they do not have enough money to pay, governments are forced to take care of it. In some countries, legislation has been created to fund the rehabilitation of abandoned mines.

Before environmental regulations existed, land used for mining was left without any remediation effort whenever mine extraction activities were completed and without a full understanding of the environmental impact. Very few countries had government mine regulations and reclamation programs until the latter part of the twentieth century. It is estimated that there are more than a million of abandoned mines worldwide, including the shafts, adit, and alluvial working mines.

Abandoned mines can cause several health problems and environmental hazards threats such as the accumulation of hazards and explosive gases resulting from the lack of circulating air in galleries, or unsafe structures that can cause unstable conditions in that area resulting in several accidents. The use of these mines for residential or industrial dumping also poses a danger from unsanitary conditions. Ingestion of dust from old waste disposal sites may lead to adverse health effects.

Mining often stripped away the vegetation and the topsoil needed to reclaim the site when all mining operations cease. The area is unproductive and unable of sustaining plant and animal life. The erosion of bare soil causes the obstruction of channels as it is carried away to nearby streams. This reduces fish habitat and interferes with natural flow patterns. Soils and water contaminated with heavy metals or chemicals may pose great risk to wildlife as they can become increasingly concentrated in animals higher up the food chain, which can cause their death, or they may become unable to reproduce.

Land degradation from old mines is well-known in almost all countries. There are many references to environmental damage from these sites. However, few systematic surveys and impact assessments have been carried out and consequently the true extent of the problem is unknown.

Mining Health Impacts

Health Definition

It is the duty of government and authorities to provide citizens with precise and appropriate information so they can protect themselves. In 1967, the World Health Organization (WHO) defined health as "a state of complete physical, mental and social well-being and not the absence of disease or infirmity." Later in 1984, the WHO redefined the concept to "the extent to which an individual or a group is able, on the one hand, to realize aspirations and to satisfy needs, and on the other, to change or cope with the environment."

Human health depends in an essential way on the environment as both a source of resources and a deposit for wastes. Environmental impact assessment is a crucial tool for maintaining and improving environmental quality while carrying out economic development. Environmental impact assessment involves the determination of changes or impacts that a project or an action will have on the surroundings (positive or negative effects) before it is carried out to prevent irreversible damages.

Environmental Impacts

Mineral resources were developed worldwide for many centuries with little environmental control. This is largely because environmental impact was not understood or appreciated as it is nowadays and the technology available at that time was not always able to prevent or control environmental damage. Some types of environmental impacts are altered landscape, contaminated soils and aquatic sediments, subsidence, and changes in vegetation. Results of theses impacts include loss of productive land, loss or degradation of groundwater, pollution of surface water, and air pollution from dust or toxic gases.

Water pollution problems by mining include acid mine drainage (AMD), metal contamination, and increased sediment levels in streams.

One of the most important environmental impacts is acid drainage (acid rock drainage – ARD or acid mining drainage – AMD). Exposure of sulfide minerals to air and humidity causes oxidation, leading to sulfuric acid formation, which can persist for hundreds of years. Highly acidic water solubilizes heavy metals (aluminum, arsenic, cadmium, copper, lead, nickel, and zinc) carrying them into streams and rivers. This will cause a major problem either because the consumption of fish where these heavy metals have bioaccumulated or because drinking water is not treated in such a way to eliminate these elements.

Mining operations can affect groundwater quality in several ways. Dissolved pollutants at a mine site are primarily metals but may include sulfates, nitrates, and radionuclides and they can migrate from mining operations to local ground and surface water. Dissolved metals can include heavy metals, which are of great concern for public health as they cause several adverse health problems including cancer. Some of the most toxic metals found in the environment near mine sites, their sources, and effects are presented in Table 2.

Two of the most toxic heavy metals that can be found in contaminated waters are arsenic and cadmium.

Arsenic is a carcinogenic metalloid that bioaccumulates in algae, crustacean, and fish. It has been ranked highest in priority on a list of top 20 hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR), and it has been classified as a known human carcinogen by International Agency for Research on Cancer (IARC) in 1987. Arsenic is possibly the only human carcinogen for which there is sufficient evidence of carcinogenic risk both by inhalation and ingestion. Arsenic exposure in mining areas mainly occurs through consumption of contaminated water and food (especially seafood). Many cases of acute and chronic arsenic poisoning have been reported worldwide. Symptoms of acute poisoning usually occur within 30 min of ingestion and may result in gastrointestinal discomfort, vomiting, coma, and sometimes even death. Chronic poisoning may result in anemia, leucopenia, skin cancer, and other internal cancers, leading in severe cases to death.

Cadmium is a metal with high toxic effects, which bioaccumulates in human kidney and liver. In 1993, the IARC has determined that cadmium is carcinogenic to humans. Cadmium is number 7 in the priority list of hazardous substances by ATSDR. Acute inhalation exposure to high levels of cadmium may result in adverse effects on lungs, such as bronchial and pulmonary irritation. Chronic inhalation or oral exposure to cadmium results in severe effects on the kidney, liver, lung, bone, immune system, blood, and nervous system.

Mining processes can also result in the contamination of sediments in streams when dissolved pollutants are discharged to surface waters. Fine grain waste materials eroded from mines can become sediments. Sediments, resulting from increased soil erosion, cause siltation or the smothering of streams beds. This siltation affects fisheries, swimming, domestic water, irrigation, and other uses of streams. Some toxic constituents associated with discharges from mining operations (i.e., lead and mercury) may be found at elevated levels in sediments. Contamination of sediments can affect human health through the consumption of fish or other biota that bioaccumulate toxic substances. Sediment contamination provides a long-term source of pollutants through potential redissolution in the water column. This may lead to chronic contamination of water and aquatic organisms.

A very important source of environmental contamination is the extraction of uranium. After the removal of high-grade uranium, an enormous volume of hazardous wastes is originated and left behind. Tailings containing most of the radionuclides species are disposed in open-air piles. Radionuclides and toxic chemicals then become readily available for dispersal through the hydrologic and atmospheric processes. Additionally, tailings release radioactive radon gas into the environment. There are several studies demonstrating that the exposure to these substances may increase the risk of developing several cancers.

Cyanides are other element of great matter in environmental contamination by mining activities such as gold extraction. They include several types of inorganic and organic compounds that contain the CN^- radical, in which a carbon atom and a nitrogen atom are linked by a triple bond. Cyanide toxicity is related to the form in which it occurs. Free cyanide is the most toxic of all the forms. Hydrocyanic acid is the most toxic of the simple compounds, and penetrates quickly in the organism and acts by attaching itself to ferric iron (Fe^{3+}). As this ion plays an active role in various enzyme systems, deactivation by cyanide of some of those systems would cause important adverse effects in the organism. Ingestion or inhalation causes headache, chest pain, and vomiting. Ingestion of a fatal dose is followed by respiratory difficulty, arrhythmia, and coma.

Mine waste deposition on soils is another important issue as it poses a risk to soil microbes, which are extremely important to nutrient cycling. These organisms are primary decomposers of soil organic matter converting nutrients into higher trophic levels.

Particulate matter is released in surface mining when overburden is stripped from the site and stored or returned to the pit. When the soil is removed, vegetation is also removed, exposing the soil to the weather, causing particulates to become airborne through wind erosion and road traffic. Particulate matter can be composed of toxic materials such as heavy metals like arsenic, cadmium, and lead. In general, particulates affect human health adversely by contributing to illness relating to the

Table 2 Main toxic metals found in mining environments, main sources, and effects

Metal	Environmental sources	Environmental effects	Human sources	Health effects
Arsenic	Arsenic can be found naturally on earth in small concentrations. May enter air, water, and land through windblown dust and water runoff. Large amounts of arsenic end up in the environment and in living organisms due to volcanoes, microorganisms, and human activities such as mining and agriculture.	It cannot be destroyed once it enters the environment. Large amounts added can spread and cause adverse health effects to humans and animals. High concentrations of arsenic may be found in plants as they absorb arsenic easily. Plant-eating freshwater organisms accumulate arsenic in their bodies and may affect the animals higher up the food chain.	Humans can be exposed through food (fish and seafood), water, and air. It may also occur through dermal contact with soil or water. Exposure may be higher for people who work with arsenic compounds, drink significant amounts of wine, smoke, and live in houses containing conserved wood of any kind and for those living on farmlands where arsenic-containing pesticides have been applied in the past.	Arsenic is one of the most toxic elements. Exposure to it can cause several health effects, namely, irritation of the stomach, lungs and intestines, decreased production of red and white cells, and skin changes. Significant uptakes of inorganic arsenic can lead to cancer development, especially skin, lung, liver, and lymphatic cancers. Higher exposure can cause infertility and miscarriages with women, skin disturbances, declined resistance to infections, heart disruptions, and DNA damage. High exposure to organic arsenic can cause nerve injury and stomachaches.
Cadmium	Cadmium can mainly be found in the earth's crust. Large amounts of cadmium are naturally released in the environment, namely, in rivers through weathering of rocks, into air through forest fires and volcanoes. Human activities such as mining also release significant amounts of cadmium in the environment. Soils are the main final destination of the industrial cadmium wastes. Other important source of cadmium in soils is the appliance of artificial phosphate fertilizers in farmlands. Waste combustion and burning of fossil fuels are a main source of cadmium in the air. Only small amounts of cadmium are released in water through disposal of wastewater from households and industries.	Acidified soils enhance cadmium uptake by plants and this causes a potential danger to the animals, which feed on them, and to the rest of the food chain. Earthworms and other essential soil organisms are extremely susceptible to cadmium poisoning. High concentrations of cadmium in soils can threaten the whole soil ecosystem. Cadmium may bioaccumulate in several aquatic organisms (mussels, oysters, shrimps, lobsters, fish, etc.). Saltwater organisms are known to be the most resistant. Animals exposed to cadmium sometimes get high blood pressures, liver disease, and nerve or brain damage.	Human uptake of cadmium occurs through food ingestion (liver, mushrooms, shellfish, mussels, cocoa powder, and dried seaweed). Exposure to high concentrations of cadmium takes place through tobacco smoke. Cadmium is transported into lungs and then distributed through the whole body. Other high exposures occur with people who live near hazardous waste sites or factories that release cadmium into the air and people who work in refinery industry.	Breathing in cadmium can severely damage the lungs and, in last instance, can cause death. Cadmium is transported to the liver and there it is bound to proteins to form complexes that are transported to the kidneys. There, it accumulates and damages filtering operations causing the excretion of essential proteins and sugars from the body. Excretion of bioaccumulated cadmium from the kidney takes a long time. It may cause damages in liver, diarrhea, stomach pains, and severe vomiting, bone fracture, reproductive failure, and possibly even infertility, damage to the central nervous and immune system, psychological disorders and it may also cause DNA damage leading to cancer development.

(*Continued*)

Table 2   Continued

Metal	Environmental sources	Environmental effects	Human sources	Health effects
Copper	Copper is a very common substance that occurs naturally in the environment. Copper is widely used and it is applied mainly in agriculture and mining industry. The production of copper has increased in past decades and consequently large amounts of copper ends up in the environment. Natural sources of copper are, for instance, windblown dust, decaying vegetation, forest fires, and sea spray. Copper compounds mostly settle and bind to sediment or soil particles. Most water-soluble copper compounds occur in the environment after application in agriculture. It can travel long distances in surface water, either suspended on sludge particle or as free ions.	Copper can accumulate in plants and animals, as it does not break down in soils. Soils with high amounts of cooper, for example, near copper-disposing factories, do not present much plant diversity. Effects on plants can cause serious problems to farmlands, which still apply copper-containing manures. If farmland soils become polluted with copper, animals like sheep may be at risk and may cause repercussions in all food chain. Copper negatively influences the activity of microorganisms and earthworms. This may severely slow down the decomposition of organic matter.	Copper can be found in food, drinking water, and air. The absorption of this element is essential since it is a trace element that is crucial for human health. Human can handle large concentrations of copper but extremely high doses can cause problems. Exposure to copper through breathing is negligible as it occurs in low concentrations but people who live near smelters that process copper ore into metal may experience this kind of exposure. People living in houses with copper plumbing are exposed to higher levels of copper through the release of this substance in drinking water by pipe corrosion. Soluble copper compounds are major threat to human health.	Some of the long-term effects are irritation of the nose, mouth, and eyes and it causes headaches, stomachaches, dizziness, vomiting, and diarrhea. High uptakes of this substance can cause liver and kidney damage and even death. There is no sufficient evidence that copper is carcinogenic. Exposure to copper fumes, dust, or mists may result in metal fume fever with atrophic changes in nasal mucous membranes. Chronic poisoning results in Wilson's disease, which is characterized by hepatic cirrhosis, brain damage, demyelination, renal disease, and copper deposition in the cornea.
Lead	Lead occurs naturally in the environment but most of what is found results from human activities such as mining and agriculture. Lead is a main constituent of several materials like ancient water pipes, lead–acid batteries, and television screens. Leaded gasoline is a major source originating lead salts. Other sources are solid waste combustion and industrial processes. The larger particles fall into the ground polluting soils or surface waters. The smaller ones travel long distances and remain in the atmosphere until it rains.	Lead accumulates in the body of water and soil organisms, which suffer severe health effects from poisoning. Shellfish experience health effects at very small concentrations. Phytoplankton is an essential source of oxygen production and many larger sea animals feed on it. If their body functions are disturbed, global balances are negatively affected. Soil organisms and consequently soil functions are also affected by lead poisoning, especially those near highways and farmlands. Lead is a truly dangerous threat as it accumulates not only in individual	Lead can enter human body through various routes, such as uptake of contaminated food, water, and air. Fruits, vegetables, meats, seafood, soft drinks, and wine may contain large amounts of it. Drinking water can become contaminated through corrosion of pipes especially and if is slightly acidic. Public water treatment systems are now required to perform pH adjustments in drinking water. Cigarette smoke also contains lead but in small amounts.	Some of the most relevant health effects in humans are the disruption of the biosynthesis of hemoglobin leading to anemia, high blood pressure, kidney and brain damage, miscarriages and subtle abortions, sperm damage, which will cause declining fertility in men, disruption of nervous system, reduced learning capacities and behavior disturbance in children. Fetuses can be severely affected by lead once it passes through the placenta causing serious damages to their nervous system and brain.

	Lead can end up in water and soils through corrosion of leaded pipelines and leaded paints. It cannot be broken down only converted in other forms.	organisms but also in entire food chains.		
Mercury	Mercury can be found naturally in the environment but rarely occurs free. It can be found in metal form, such as salts or organic compounds. It enters the environment from normal breakdown of minerals in rocks and soils through exposure to wind and water. Human activities highly increase mercury levels in the environment, namely, in air through fossil fuel, mining, and smelting. The application of agricultural fertilizers and the disposal of industrial wastewater release mercury into soils and water. It is extremely used in thermometers and barometers, recovery of gold from ores and more. Acid surface waters contain much higher mercury concentrations as it is mobilized from the ground.	Microorganisms can convert mercury present in surface waters and soils into methyl mercury, which may be quickly absorbed by many organisms. Fishes absorb and accumulate great amounts of this compound, which may result in the accumulation in food chains. Some of the effects on animals are kidney and intestines damage, stomach disruption, reproductive failure, and DNA alteration.	Mercury is not usually found in food, but it can enter food chains trough smaller organisms that bioaccumulate it, and which are consumed by man. Mercury can enter the human body through the consumption of plants to which mercury-containing sprays had been applied. It is used in several household products such as barometers, thermometers, and fluorescent light bulbs. If some of those broke, high exposure can happen through breathing while it vaporizes.	High exposure to mercury vapors causes harmful effects like brain and kidney damage, lung and eye irritation, skin rashes, vomiting, and diarrhea. Other effects of mercury exposure are disruption of nervous system, DNA and chromosomal damage, negative reproductive effects like sperm damage, birth defects, and miscarriages.
Nickel	Nickel occurs in the environment at very low concentrations. It usually occurs in ores, which are mined in various countries worldwide. It is applied as a component of steel and other products, including jewelry. Nickel is released into the air by power plants and trash incinerators. It settles to the ground or falls down with rain. Nickel can also end up in surface waters when present in wastewater streams. Most nickel released in the environment becomes immobile as it is adsorbed by sediment or soil particles. In acid soils, it becomes more mobile and often runs off to groundwater.	High concentrations of nickel on sandy soils can severely damage plants and on surface waters it can reduce the growth rates of algae. Microorganisms are also affected, but they generally become quickly resistant to nickel. Nickel is an essential element for animals at low concentrations. It is extremely harmful when the maximum tolerable amount is exceeded as it can cause different kinds of cancer, especially in those organisms living near refineries.	Humans can be exposed to nickel by breathing contaminated air, drinking contaminated water, eating contaminated food, or smoking cigarettes. Dermal exposure can also occur with contaminated soils and waters. Foodstuffs usually have small amounts of nickel. Chocolate and fats are an exception as they contain higher quantities. Smokers have high uptake of nickel through cigarette smoke.	Nickel is an essential element in small amounts but when the uptake is too high it can cause severe damages to human health. Some of the most important effects are increased chances of developing lung, nose, larynx, and prostate cancer, lung embolism, respiratory failure, birth defects, asthma and chronic bronchitis, and heart disorders. Nickel exposure through breathing can cause pneumonitis as nickel fumes are respiratory irritants. Sensitive individuals may develop dermatitis known as 'nickel itch' after exposure to nickel and its compounds.

Table 2   Continued

Metal	Environmental sources	Environmental effects	Human sources	Health effects
Uranium	Uranium can be found naturally in very small amounts in rocks, soil, air, and water. It is a radioactive element that is very reactive so it cannot be found in the environment in its elemental form. Human activities increase uranium quantity through mining and milling processes. Erosion of tailing from mines and mills can cause high amounts of uranium to be released into the environment. Uranium in air exists as dust that falls into surface water, plants, or soils through settling or rainfall. Uranium compounds in soil combine with other compounds and they can persist there for many years. It has several applications, namely, in nuclear energy, radiometric dating, and fertilizers. Uranium itself is not particularly dangerous, but some of the decay products like radon pose a real threat to all living organisms.	Water containing small amounts of uranium is generally safe to drink. It does not accumulate in fish or vegetables and it is quickly eliminated from the organism through urine and feces. Plants absorb uranium through their roots and keep it there. Therefore, root vegetables may present higher amounts of this element but when they are washed uranium is removed.	Humans may experience exposure to uranium through food, air, soil, and water, as it is naturally present in all of them. This kind of exposure is extremely low causing no harm to human life. Higher exposure can be experienced by people living near hazardous waste sites like mining areas, working in the phosphate industry, eating crops that grow in contaminated soils, and people who drink water from a uranium waste disposal point. Artists that are still using uranium glazes also experience higher exposure.	Uranium is a radioactive substance and therefore much research has been made to identify its major health effects. Exposure to natural levels of this element causes no harm for human life. Exposure to higher levels can cause severe health effects such as kidney disease. People exposed for a long period of time to uranium radionuclides formed during radioactive decay may develop cancer. Enriched uranium is the most harmful form as it is the most radioactive. This form can end up in the environment during accidents in nuclear power plants. People exposed to it may develop cancer within a few years.

respiratory tract, such as emphysema, but they can also be ingested or absorbed into the skin.

Gaseous emissions are also important since some gaseous emissions like sulfur oxide affect the downwind environments through acid precipitation or dry deposition. Some metals like arsenic, zinc, mercury, and cadmium vaporize when heated in pyrometallurgical processes. If they are not captured and condensated during these processes, they will escape to the surrounding environment.

Finally, mining can cause physical disturbances to the landscape, such as waste rock piles and open pits. These disturbances may contribute to the decline of wildlife and plant species in the area. It is possible that many of premining surface features cannot be replaced after mining closure. The instability of mining structures is a very important issue. For instance, mining subsidence (i.e., ground movements due to the collapse of overlying strata into voids by ground mining) can cause damage to buildings, pipelines, and roads.

Most studies report several adverse effects caused by mining wastes at all levels of organization form cellular to ecosystem level. These studies were able to establish a casual relationship between mining activities and the accumulation of trace elements at concentrations that are potentially harmful to all organisms' health.

Community Impacts

The impact of mining activities on the community health occurs at various levels, namely, adverse health effects that result from environmental exposure to contaminated air, water, soil, and noise pollution; and nonenvironmental effects such as mining disasters and pit closure, which can affect the community directly and indirectly.

Mining has social and emotional impacts, with negative effects on the quality of life and lifestyle. It can be manifested through physical and emotional sickness and several changes in the behavior of all community members. Mining comes along with the promise of wealth and jobs, but it can also bring negative social effects such as appropriation of lands belonging to the local communities, alteration of social relationships, and destruction of forms of community subsistence and life.

Migration is another important issue. For example, in South Africa gold mining involves workers coming from neighboring countries. If a disaster occurs, a pit closures or even if some workers become incapacitated from the contraction of communicable diseases, namely, HIV/AIDS, the community affected reaches international scale.

Few research studies have been conducted on the exposure and relative risk for residents living in the area of a mine when compared to the number of studies on occupational exposure. Existing studies point to several adverse effects on communities' health such as

mesothelioma and respiratory illness, but most of the results are conflicting. It is worth noting that many adverse effects are only noticed in risk groups such as children and elder people, since their health is more likely to be affected, and some effects are only noticed many years later.

Occupational Impacts

Mining remains one of the most hazardous occupations in the world, both in terms of short-term injuries and fatalities, but also due to long-term impacts. Noise, vibration, dust, heat, and humidity are some of the factors that affect the ability to work efficiently. Health effects are related to different scales of mining operation (small and large), different types of mine (open cast and deep), and minerals (asbestos, coal, gold, and uranium).

Workers of deep mines are exposed to more hazardous situations than those working in open cast mines, due to the risk of collapse, poor air quality, and underground explosions.

Small-scale mines are more hazardous than large-scale mines in what concerns to fatalities and injuries. This is because they are mainly surface mines or small-scale operations that employ younger and inexperienced individuals.

Major impacts on workers' health are cancer and respiratory diseases such as asbestosis, silicosis, and pneumoconiosis. Cancer is long-term impact and it is one of the most important occupational health impacts as miners can develop health problems many years after they finish working. Long-term effects are particularly prominent in miners who have worked in asbestos, coal, and uranium mines, or in miners who have been exposed to a mixture of different silica and other dusts (copper, gold, and zinc mines).

Injuries have declined in importance since safety in mines has improved with time but continue to be an important issue. Number of fatalities in the United States has dropped from 266 in 1979 to 55 in 2004 (**Figure 3**) and the number of injuries show a significant decline over time (**Figure 4**). Even with the considerable reduction in mining injuries and fatalities, both the number and severity of mining accidents occurring are still unacceptable. The data from coal mines reveal 346 446 injuries from 1978 through 2006 (11 946 per year), and 331 947 injuries in the metal and nonmetal sector (11 446 per year). The total number of fatalities for the same period was 1,882 in coal mining (65 per year) and 1,650 for metal and nonmetal mining (57 per year).

Mental health is another long-term effect particularly noticeable in isolating mining communities and after pit closure. Unemployed miners show higher rates of psychological distress and morbidity compared to working miners and workers in other professions.
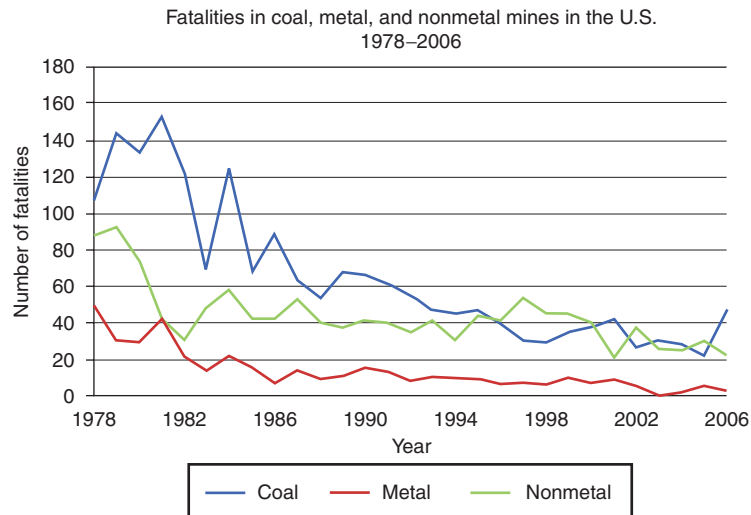
Fatalities in coal, metal, and nonmetal mines in the U.S.
1978–2006

Figure 3   Graphic representation of the number of fatalities in coal, metal, and nonmetal mines in the United States between 1978 and 2006.

Injuries in coal, metal, and nonmetal mines in the U.S.
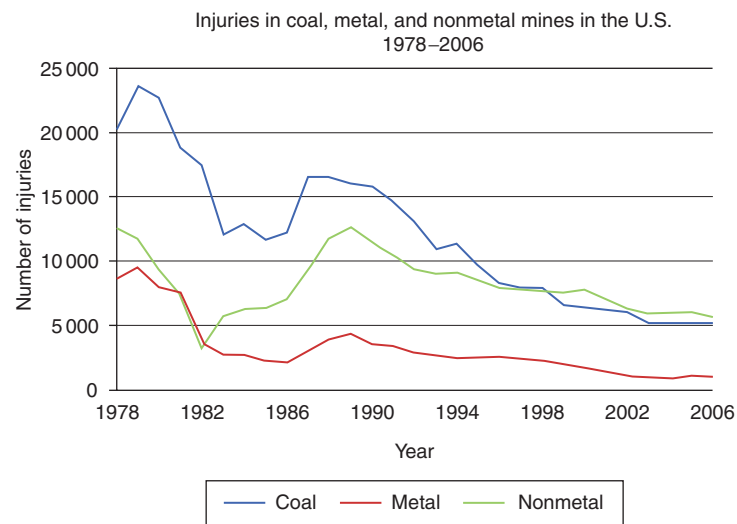1978–2006

Figure 4   Graphic representation of the number of injuries in coal, metal, and nonmetal mines in the United States between 1978 and 2006.

It is important to further improve miners' safety and health by using the most effective approaches possible.

Initiatives to Reduce Mining Impacts

During recent decades, public concern about the protection, preservation, and restoration of the environment has increased. Toward this direction, legislative action on environmental issues that affect the mining industry has been taken worldwide.

Environmental and Community Initiatives

Many millions of dollars have been spent by the mining industry to develop techniques to extract even small concentrations of mineral from the earth but very little has been spent to put the land back together with no harm for living organisms. Very few resources have been wasted to study the impact of mining activities on communities' health. Nowadays people continue to be exposed to toxic levels of pollution.

Investment in improving the health of communities impacted by mining activities serves not only to decrease the current exposure and risk but also to demonstrate the need for changes in mining laws and regulations.

Long-term health impacts related to mining activities remain long after the company is gone, and there are few evidences that companies are willing to address these long-term responsibilities. Health impacts related to

mine products are more the responsibility of the industry than any other health program.

New efforts to reform modern mining and adequate cleanup programs for abandoned mines offer a time opportunity to protect people's health and the entire food chain. This protection benefits not only those who live closest to mines but everyone who is affected.

Mining companies have recently started to put health programs into place around mines. Some experienced mining engineers are working with environmental and community organizations to identify best possible reclamation options.

Appropriate environmental laws with adequate monitoring enforcement have been adopted to prevent most of the damage caused in mine communities.

A process of population study known as molecular epidemiology has been developed to integrate laboratory measurements of internal dose, biologically effective dose, biologic effect, and susceptibility with epidemiological methodologies, thus linking individual exposure with an important biological event. The biological component is evaluated with biological markers, also called biomarkers, which are observable end points that indicate events in the process leading up to disease. Risk assessment should include the measurement of actual impacts on biological end points from trace element contamination in soil, surface water, groundwater, air and sediments at various levels of biological organization. By providing risk assessment data, these studies will have impact on regulatory policies and on establishing disease prevention strategies.

Occupational Initiatives

After a long period of contradiction and struggle, health and safety improvements in mines have been developed. Many initiatives have been taken in the past decades to improve occupational health and safety mainly by workers, unions, nongovernmental organizations (NGOs), governments, and the industry itself. Past improvements in working conditions were made by mining companies that were involved in programs of worker's health and safety. Such improvements included safety measures to reduce injuries or air conditioning thus reducing heat stress. Nowadays, they also include programs to assist miners in their living and social conditions. Unions have had an essential role in the development of safer conditions in mines, and in the development of health information and other sponsored services. The effective use of the law to protect workers has been of crucial importance to improve mining conditions.

A much more scientific consciousness has developed in the past decades. All involved in mining activities now understand the long-term impacts. Workers have been able to use scientific evidence for improving hazard exposure and for changing health and safety legislation.

Legislation

There are some major laws and regulations involving the mining industry. One of the most important is the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA), generally known as Superfund, was enacted in 1980. This law oblige companies to report releases of hazardous substances to the environment and requires cleanup of hazardous sites. This program was established to place, investigate, and clean up the worst abandoned hazardous waste sites nationwide and is currently being used by the U.S. EPA to clean up mineral contamination at numerous locations.

Essential regulations are the Federal Water Pollution Control Act (commonly known as the Clean Water Act), the Resource Conservation and Recovery Act (RCRA), and the National Environmental Policy Act (NEPA).

The former came into effect in 1977 and requires mining operations to meet standards for surface water quality and for controlling discharges to surface water. The RCRA was enacted in 1976 and regulates the generation, storage, and disposal of solid waste and hazardous waste. These wastes are managed from the point of generation to disposal. Finally, NEPA was enacted in 1970 and requires federal agencies to prepare Environmental Impact Statement (EIS) for major federal actions that may significantly affect the environment.

The European Union (EU) has developed some environmental directives that have had a significant effect on the mining industries of member nations. Each country's environmental laws derive from these directives. Some of the most important ones are the Environmental Impact Assessment Directive (similar to the EIS), the Water Framework Directive (similar to the Clean Water Act), and the Waste Framework, Hazardous Waste, and Landfill Directives (similar to the RCRA).

More recently, the EU has introduced measures to prevent or minimize any adverse effects on the environment and the health risks resulting from the management of waste from the extractive industries, such as tailings and displaced material (Directive 2006/21/EC of the European Parliament and of the Council of 15 March 2006 on the management of waste from extractive industries). This directive applies to waste resulting from the extraction, treatment and storage of mineral resources, and the working of quarries.

All these programs are trying to solve the problems associated with mining, and they have similarity to other programs worldwide.

Recent regulations are emphasizing the responsibility and liability of mine administrators, and require that they

manage health and safety to provide a safe place to work. The long-term goal should be that individuals could work all life in a mine and continue to be healthy and unharmed. Governments, which ratified Convention 176 – Safety and Health in Mines, 1995 – committed to adopt legislation for its implementation, including the provision for inspection services and the designation of the competent authority to monitor and regulate the various aspects of occupational safety and health (OSH) in mines. The convention also delineates measures for reporting and investigating disasters, accidents and unsafe events related to mines, and for the compilation of the significant statistics.

Legislation should provide an efficient support of standards and directions. Regulatory authorities responsible for administering that legislation need to do so in a professional, constructive, and consistent way.

Future Directions

The need to address public health and ecological threats posed by mining pollution should be given a priority. Global initiatives should be developed to significantly reduce the amount of hazardous material released into the environment and to apply efficient measures to assure there will be no health impacts resulting from mining activities, especially from proposed new mines.

Cleanup and remediation measures are crucial since abandoned mines will continue to have an impact on both health and environment unless mine closure practices are strictly preformed. Therefore, technical expertise in terms of the response actions, technology types, and remediation and cleanup alternatives would be needed for this purpose as well as appropriate funding.

It is vital to conduct regular monitoring and surveillance activities of the environment and community's health. Information from this monitoring should be provided in an accessible and comprehensive form to the general public.

It is important to have more openness and transparency within the mining sector particularly in undeveloped countries of South America, Asia, and Africa. There is a further need for a deeper and long-term evaluation of the mining impacts on workers and communities' health.

It is also important to develop new interventions and control strategies for the risks associated with mining equipment, to train miners, and to identify and implement interventions that address the remaining sources of risk.

All these measures will help to better protect the health and safety of people working in, living near, and those otherwise impacted by historic, current, and proposed mines.

See also: Mineral and Fuel Extraction: Health Consequences.

Further Reading

Boulanger A and Gorman A (2004) *Hardrock Mining: Risks to Community Health*. Bozeman, MT: Women's Voices for the Earth (available online at www.earthworksaction.org/pubs/MiningHealthReport.pdf).

Bridge G (2004) Contested terrain: Mining and the environment. *Annual Reviews of Environment and Resources* 29: 205–259.

Coelho P, Silva S, Roma-Torres J, et al. (2007) Health impact of living near an abandoned mine-Case study: Jales mines. *International Journal of Hygiene and Environmental Health* 210: 399–402.

Donoghue AM (2004) Occupational health hazards in mining: An overview. *Occupational Medicine* 54: 283–289.

EPA (2000) *Abandoned Mine Site Characterization and Cleanup Handbook*. Seattle: U.S. Environmental Protection Agency.

Groves WA, Kecojevic VJ, and Komljenovic D (2007) Analysis of fatalities and injuries involving mining equipment. *Journal of Safety Research* 38: 461–470.

Health Canada (2004) *Canadian Handbook on Health Impact Assessment* (4 vols.) A Report of the Federal/Provincial/Territorial Committee on Environmental and Occupational Health. Ottawa: Health Canada.

Hermanus MA (2007) Occupational health and safety in mining status, new developments, and concerns. *The Journal of the Southern African Institute of Mining and Metallurgy* 107: 531–538.

IARC (1993) *Cadmium and Certain Cadmium Compounds. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans,* vol. 58: Beryllium, Cadmium, Mercury and Exposures in the Glass Manufacturing Industry. Lyon: IARC press.

IARC (1987) *Arsenic and Arsenic Compounds. IARC Monographs on the Evaluation of Carcinogenic Risk to Humans. Supplement 7. Overall Evaluations of Carcinogenicity: Updating of IARC Monographs Volumes 1–42*. Lyon: IARC press.

Jennings NS (2001) *Improving Safety and Health in Mines: A Long and Winding Road?* Paper prepared for MMSD. London: IIED and WBCSD.

Jones FT (2007) A broad view of arsenic. *Poultry Science* 86: 2–14.

NIOSH (2007) *Mining Safety and Health Research at NIOSH: Reviews of Research Programs of the National Institute for Occupational Safety and Health*. Washington D. C.: The National Academies Press.

Peplow D and Edmonds R (2005) The effects of mine waste contamination at multiple levels of biological organization. *Ecological Engineering* 24: 101–119.

Stephens C and Ahern M (2002) *Worker and Community Health Impacts Related to Mining Operations Internationally. A Rapid Review of the Literature*. Paper prepared for MMSD. London: IIED and WBCSD.

Relevant Websites

http://www.cdc.gov/niosh/
    Department of Health and Human Services, Centers for Disease Control & Prevention, The National Institute for Occupational Safety and Health.
http://www.ilo.org/global/lang-en/index.htm
    International Labour Organization.
http://www.msha.gov
    United States of America Department of Labour Mine Safety and Health Administration.
http://www.epa.gov
    United States of America Environmental Protection Agency.
http://www.who.int/en/
    World Health Organization.

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