



Immunological alterations in individuals exposed to metal(loid)s in the Panasqueira mining area, Central Portugal



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HIGHLIGHTS

- Immunotoxic effects were observed in both exposed populations.
- Effects were associated to metal(loid) contamination derived from mine activities.
- Competent authorities need to intervene and help protect exposed populations.

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ABSTRACT

Environmental studies performed in Panasqueira mine area (central Portugal) identified high concentrations of several metal(loid)s in environmental media, and individuals environmentally and occupationally exposed showed higher levels of As, Cr, Mg, Mn, Mo, Pb and Zn in blood, urine, hair and nails when compared to unexposed controls. To evaluate the presence of immunological alterations attributable to environmental contamination, we quantified neopterin, kynurenine, tryptophan, and nitrite concentrations in plasma, and analysed the percentage of several lymphocytes subsets, namely CD3⁺, CD4⁺ and CD8⁺ T-cells, CD19⁺ B-cells, and CD16⁺56⁺ natural killer (NK) cells in a group of individuals previously tested for metal(loid) levels in different biological matrices. The environmentally exposed group had 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. Analysed biomarkers were found to be influenced by age, particularly neopterin, kynurenine and kynurenine to tryptophan ratio (Kyn/Trp) with significantly higher levels in older individuals, and %CD3⁺, %CD8⁺ and %CD19⁺ with significantly lower values in older individuals. Males environmentally exposed showed significantly lower values of %CD19⁺ when compared to control females. The concentration of Pb in toenails was associated to the level of neopterin, kynurenine and Kyn/Trp ratio (all direct), and the concentration of Mn in blood to the level of %CD8⁺, %CD19⁺ (both inverse) and CD4⁺/CD8⁺ ratio (direct). Overall our results show that the metal(loid) contamination in Panasqueira mine area induced immunotoxic effects in exposed populations, possibly increasing susceptibility to diseases.

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1. Introduction

Mining activities can adversely affect the health of miners and communities living near mining sites (Coelho et al., 2011b). Several environmental studies performed in the Panasqueira mine in central Portugal showed high concentration of toxic metals and metalloids [= metal(loid)s] in stream sediments, superficial and ground waters from local water courses, road dust, soils, and plants for human consumption cultivated in nearby villages (Ávila et al., 2008; Ferreira

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da Silva et al., 2013; Grangeia et al., 2011; Salgueiro et al., 2008). In order to evaluate the effect of this contamination on populations living nearby and/or working in the mine, biological samples were collected and analysed to quantify the levels of several metal(loid)s – arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), sulphur (S), selenium (Se), silicon (Si) and zinc (Zn) (Coelho et al., 2013). Results showed that exposed populations had significantly higher concentrations of As, Cr, Mg, Mn, Mo, Pb and Zn when compared to unexposed controls. Subjects with the highest concentrations of metal(loid)s were those environmentally exposed, specifically females who presented significantly higher concentrations of the most toxic elements – As, Cr, Mn and Ni.

Most metal(loid)s are toxic to living organisms, even those considered as essential can be toxic when in excess and may cause major health effects, i.e., development retardation, endocrine disruption, kidney damage, immunological and neurologic effects, and several types of cancer (Mudgal et al., 2010). The continuous disturbance of important biochemical processes, such as redox homeostasis, can be associated with chronic pro-inflammatory signalling, leading to induction of proto-oncogenes and/or anti-apoptotic factors, causing a persisting overstimulation of the immune system, thus leading to immunotoxicity (Henkler et al., 2010).

Immunotoxicity is 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, although reported effects in exposed populations are conflicting (Cabassi, 2007). This may be mostly attributed to the limited specificity of biomonitoring studies, often not considering the toxicity of the specific element, its concentration, route of exposure, duration of exposure and biologic availability (Lehmann et al., 2011).

Biological parameters measured to assess human immunotoxicity include the count of blood components, antibody-mediated immunity (serum concentrations of immunoglobulins), phenotype analysis of lymphocytes by flow cytometry, among others. More recently Capuron et al. (2009) showed that increased concentrations of inflammatory markers were associated with reduced quality of life in elderly persons, cognitive decline and mood disorders. These alterations influence the metabolism of neurotransmitters and neuroendocrine functions involved in the development of several behaviour symptoms known as sickness behaviour (Capuron et al., 2011). The metabolism of some of these neurotransmitters (e.g. serotonin, norepinephrine, and dopamine) which are synthesized within the brain from their precursors – tryptophan and tyrosine – can be affected by immune activation (Capuron et al., 2011; Schroecksnadel et al., 2006).

Preliminary results from the analyses performed in a small group of individuals living nearby and working in the Panasqueira mine pointed to immunotoxic effects experienced by exposed populations related to the contamination derived from mining activities, but a more robust study with a higher number of subjects and complementary immune markers was needed to confirm these findings (Coelho et al., 2011a, 2012).

The aim of the present study was to evaluate the immunotoxic effects associated with the environmental and occupational exposure to metal(loid) contamination in Panasqueira mine area. Levels of neopterin, tryptophan, kynurenine and nitrite, and percentage of several lymphocytes subsets – 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 selected as key immune markers. Possible relationships between the levels of immunotoxicity biomarkers and the concentrations of metal(loid)s in biological samples were investigated.

2. Material and methods

2.1. Study population

The study population consisted of a total of 122 subjects living in the Panasqueira mine district as detailed in Coelho et al. (2013). Forty-one individuals living in villages located in the proximity – within a 6 km radius – of the mine (S. Francisco de Assis and Barroca do Zêzere) were classified as environmentally exposed (16 males and 25 females), 41 males working in the Panasqueira mine and living in exposed and non-exposed villages were classified as occupationally exposed, and 40 additional subjects (17 males and 23 females) without any exposures to mining activities, or other known toxic exposure, represented the controls. This latter group included individuals living in non-contaminated areas located north-east and west of the mine (Casegas and Unhais-o-Velho, respectively). Control individuals worked mainly in administrative offices and were matched with the environmentally exposed group by age, gender, lifestyle, and smoking habit (categorized as ever/never smokers). Only individuals living in the villages for at least 5 years and aged over 18 years were selected. Health conditions, medical history, medication, diagnostic tests (X-rays, etc.), and lifestyle factors were assessed by means of a questionnaire. Subjects were also interviewed about specific symptoms related to metal(loid) exposure, the presence of chronic respiratory diseases, such as bronchitis; drinking and agricultural water source; agricultural practices, including pesticides usage; and diet. Conditions known to affect the immune system or with a significant effect on any biomarker to be analysed were considered exclusion criteria. All subjects were fully informed about the procedures and the aims of this study, and signed an informed consent form. Approval for this study was obtained from the institutional ethical board of the Portuguese National Institute of Health.

2.2. Sample collection

Blood samples were collected by venipuncture in tubes containing ethylenediamine tetraacetic acid (EDTA). Samples were transported under refrigeration and kept at approximately +4 °C for analysis of lymphocyte subsets. For quantification of neopterin, tryptophan, kynurenine and nitrite the tubes were centrifuged and the supernatant plasma was collected and stored at –20 °C until analysis. All samples were coded and analysed under blind conditions.

2.3. 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 quantified by flow cytometry as described in García-Lestón et al. (2011). Analysis was performed on a FACScalibur flow cytometer using Cell Quest Pro software (Becton Dickinson). A minimum of 10⁴ events in the lymphocytes window was acquired.

2.4. Quantification of neopterin, tryptophan, kynurenine and nitrite in plasma samples

A commercially available enzyme-linked immunosorbent assay (ELISA) kit (BRAHMS, Hennigsdorf, Germany) was used to determine neopterin concentration in plasma, following the manufacturer's instructions. The limit of detection (LOD) was 2 nmol/L neopterin. Plasma tryptophan and kynurenine concentrations were measured by a high-performance liquid chromatography (HPLC) methodology with 3-nitro-L-tyrosine as internal standard, as previously described (Widner et al., 1997). The extent of tryptophan breakdown was estimated by calculating the kynurenine to tryptophan ratio (Kyn/Trp), expressed in

μmol kynurenine per mmol tryptophan. LOD was $0.1 \mu\text{mol/L}$ tryptophan and $0.5 \mu\text{mol/L}$ kynurenine. In order to estimate nitrite oxide (NO) production, the stable NO metabolite nitrite (NO_2^-) was determined by the Griess reaction assay (Promega, Madison, Wisconsin) (Griess, 1879). LOD was $1.5 \mu\text{mol/L}$ nitrite. Variation coefficients of all the methods lied between 2 and 10%.

2.5. Statistical analysis

The three study groups were compared according to socio-demographic, i.e., gender, age, village of residence, and years of work in the mine, and lifestyle factors potentially influencing immunotoxicity, i.e., smoking habits. 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) contamination] on the multivariable analyses. The Chi-square test was applied for categorical variables and the analysis of variance (ANOVA) was applied for continuous variables. 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 data was applied to neopterin, tryptophan, kynurenine and nitrite levels, Kyn/Trp ratio and to percentages of the different lymphocyte subsets. A multiple linear regression analysis was performed to estimate the effect of exposure on the log-transformed data. The resulting statistics, i.e., mean ratio, expresses the ratio of the mean value of that specific parameter in the exposed group to the corresponding mean value in the controls; the appropriate 95% confidence interval is also provided. Adjustment for age and smoking habit was applied to all models and 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 assess the effect of metal(loid) concentration on biomarkers of immunotoxicity. The study subjects were divided into three groups according to the tertile distribution of each metal(loid). The resulting three-level factors [one factor for each metal(loid)] were, in turn, fitted to the log-transformed value of immunotoxicity biomarkers in a linear regression model which 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. Spearman rank correlation analyses were carried out between neopterin and tryptophan metabolism substances (kynurenine and Kyn/Trp).

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.

3. Results

The general characteristics of the study groups are summarized in Table 1. No significant differences in age and source of water for consumption between the three groups were observed. Difference in the distribution of gender and smoking habits were mostly due to only male composition of the occupationally exposed group, the vast majority smokers. Results concerning the level of immunotoxicity biomarkers in the study groups are presented in Table 2. 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, nitrite and Kyn/Trp ratio among the three groups.

When multivariable modelling was applied, levels of neopterin, kynurenine and Kyn/Trp ratio were found to be influenced by age (Table 3), with significantly higher mean ratios (MRs) in older individuals, when compared to the youngest group (25–50 years). 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 4). In the environmentally exposed group we observed MRs 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, MRs 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.

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

Table 1
Description of the study population from the Panasqueira mine area.

Variable	Controls	Environmentally exposed	Occupationally exposed	P-value
Total	40	41	41	
Duration of work (years, current miners) ^a	–	–	25.07 ± 7.07	
Village				
S. Francisco de Assis	0 (0%)	19 (46%)	9 (22%)	<0.001 ^b
Barroca do Zêzere	0 (0%)	22 (54%)	8 (19%)	
Unhais-o-Velho	24 (60%)	0 (0%)	24 (59%)	
Casegas	16 (40%)	0 (0%)	0 (0%)	
Gender				
Females	23 (59%)	25 (61%)	0 (0%)	<0.001 ^b
Males	17 (43%)	16 (39%)	41 (100%)	
Age (years) ^a	56.6 ± 12.58	61.71 ± 13.5	62.05 ± 7.57	0.063 ^c
Smoking habit				
Never smokers	25 (62%)	32 (78%)	16 (39%)	0.001 ^b
Ever smokers	15 (38%)	9 (22%)	25 (61%)	
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%)	

^a Mean ± standard deviation.

^b Chi-square test.

^c ANOVA test.

Table 2
Levels of immunotoxicity biomarkers in the study groups.

Variable	Controls		Environmentally exposed		Occupationally exposed		P-value ^a
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	
Neopterin (nmol/L)	24	4.4 ± 0.8	22	4.8 ± 0.8	32	4.9 ± 1.6	0.255
Tryptophan (μmol/L)	24	51.2 ± 7.7	22	52.0 ± 7.6	32	52.8 ± 8.1	0.773
Kynurenine (μmol/L)	24	1.8 ± 0.4	22	2.0 ± 0.6	32	1.9 ± 0.5	0.277
Kyn/Trp (μmol/mmol)	24	35.4 ± 6.1	22	39.3 ± 10.0	32	37.1 ± 10.1	0.361
Nitrite (μmol)	24	23.4 ± 20.5	22	16.5 ± 12.3	32	32.9 ± 32.2	0.112
%CD3 ⁺	40	75.5 ± 8.9	35	70.6 ± 10.0	38	68.9 ± 12.9	0.022
%CD4 ⁺	40	47.2 ± 6.9	35	46.6 ± 8.8	38	40.1 ± 9.8	0.001
%CD8 ⁺	40	26.0 ± 9.5	34	20.9 ± 6.3	38	26.6 ± 11.2	0.022
CD4 ⁺ /CD8 ⁺	40	2.0 ± 0.7	34	2.4 ± 0.8	38	1.9 ± 1.1	0.037
%CD19 ⁺	40	8.8 ± 6.9	35	7.7 ± 3.3	38	7.2 ± 3.6	0.339
%CD16 ⁺ 56 ⁺	40	13.7 ± 6.8	35	17.2 ± 7.7	38	20.2 ± 11.9	0.008

^a ANOVA test.

environmentally exposed group when compared to control females (MR = 0.60; 95% CI 0.37–0.98).

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.

To quantitatively evaluate the immunotoxic effect of metal(loid) exposure on the selected biomarkers, we associated these latter data with metal(loid) concentrations in the biological matrices (blood, urine, finger and toenails, and hair) measured in the same subjects in the above mentioned study (Coelho et al., 2013). Subjects were divided according to the tertile distribution of each metal(loid) level in each biological matrix. Higher MRs 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 Kyn/Trp in the 3rd tertile, whereas the MR of neopterin in the 3rd tertile was borderline significant ($P = 0.067$) (Table 5). Levels of Mn in the 2nd tertile were significantly associated with decrease in %CD8⁺ and increase in CD4⁺/CD8⁺ ratio, and in the 3rd tertile with increase in CD4⁺/CD8⁺ and decrease in %CD19⁺ (Table 6).

We also investigated the presence of differences among groups concerning the report of symptoms related to metal(loid) intoxication (Table 7). Significant differences were obtained for *cough with expectoration* and *taking medicines*, with lower MR in both exposed groups. Moreover, as expected, significant influence of smoking was observed in *cough with expectoration* (higher MR in ever smokers) and of age in *sick in recent years* and *taking medicines* (higher MR in older individuals) (data not shown).

Table 3
Effect of exposure on neopterin, tryptophan, kynurenine and nitrite concentrations stratified by exposure, age, and smoking habits. Log linear regression estimates adjusted 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]

Parameter-specific actual confounders: Neo – water consumption. No parameter-specific actual confounder was identified for Trp, Kyn, Kyn/Trp and nitrite.

* $P < 0.05$.

** $P < 0.01$.

4. Discussion

Results from environmental geochemical campaigns performed in the proximity of the Panasqueira mine showed a high degree of contamination by several metal(loid)s in different media (Ávila et al., 2008; Ferreira da Silva et al., 2013; Grangeia et al., 2011; Salgueiro et al., 2008). Accordingly, biomonitoring studies carefully designed and carried out in local populations living near or working in the mine showed significantly higher concentrations of As, Cr, Mg, Mn, Mo, Pb and Zn in blood, urine, nails and hair samples when compared to controls (Coelho et al., 2013). Preliminary data showed also immunotoxic effects in these populations (Coelho et al., 2011a, 2012). Therefore, a more robust study to evaluate the immunological alterations induced by this contamination was carried out, analysing a larger number of immunotoxicity biomarkers, including the levels of neopterin, tryptophan, kynurenine, nitrite, and percentages of several lymphocytes subsets. To our knowledge this is the first study in which these biomarkers were evaluated in human populations exposed to metal(loid)s resulting from mining activities both environmentally and occupationally.

Significant modifications of immunotoxicity parameters were observed in the exposed groups, specifically in the percentages of lymphocytes subsets (except for %CD19⁺). Lymphocytes are the primary cells involved in acquired immunity. They are highly specialized cells that interact with other cells to initiate immune response and 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

Table 4

Effect of exposure on lymphocytes subsets stratified by exposure, age, and smoking habits. Log linear regression estimates adjusted 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]

Parameter-specific actual confounders: %CD3⁺ and %CD4⁺ – fish consumption > 2 times/week; %CD8⁺ – alcohol consumption, tea consumption; CD4⁺/CD8⁺ – alcohol consumption, tea consumption, fish consumption > 2 times/week; %CD9⁺ – tea consumption, fish consumption > 2 times/week, surgery in recent years; %CD16⁺56⁺ – surgery in recent years.

* P < 0.05.

** P < 0.01.

existing evidence linking these changes to exposure to immunotoxic compounds and consequently to 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. 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⁺ ratio, while mine workers in %CD3⁺, %CD4⁺ and %CD16⁺56⁺ (Table 4). As described in Coelho et al. (2013), 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 5) and Mn in blood (Table 6). Considering all this information, our results seem to point to occupational exposure to Pb and environmental and occupational exposure to Mn as the main conditions 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., 2012). In our study high levels of Pb (2nd and 3rd tertile with mean concentrations of 0.68 and 2.43 µg/g, respectively) in toenails increased the MR of neopterin, kynurenine and Kyn/Trp. Nevertheless, when exposed populations were 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 et al. (2012), 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. (2006) 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-γ (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 ratio in serum/plasma, has been shown to strongly correlate with neopterin concentrations (Schroeksnadel 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 2). 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 (Schroeksnadel et al., 2006). Besides, neopterin concentration and Kyn/Trp ratio 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 (Schroeksnadel et al., 2006).

Table 5

Effect of the levels of Pb in toenails on neopterin, kynurenine and Kyn/Trp. Log linear regression estimates adjusted for age, smoking and immunotoxicity parameter-specific actual confounders.

Pb in toenails (mean ± SD)	Neopterin		Kynurenine		Kyn/Trp	
	MR	95% CI	MR	95% CI	MR	95% CI
1st tertile (0.32 ± 0.09)	1.00		1.00		1.00	
2nd tertile (0.68 ± 0.16)	1.10	[0.97;1.24]	1.25*	[1.07;1.47]	1.08	[0.95;1.23]
3rd tertile (2.43 ± 1.51)	1.12	[0.99;1.26]	1.34*	[1.16;1.56]	1.28*	[1.14;1.44]

Values presented for each tertile are expressed in µg/g.

Parameter-specific actual confounders: Neopterin – water consumption, cough with expectoration; Kyn/Trp – alcohol consumption, sickness in recent years, surgery in recent years, agricultural work, water consumption. No parameter-specific actual confounder was identified for kynurenine.

* P < 0.01.

Table 6
Effect of the levels of Mn in blood on %CD8⁺, CD4⁺/CD8⁺ and %CD19⁺ lymphocytes. Log linear regression estimates adjusted for age, smoking and immunotoxicity parameter-specific actual confounders.

Mn in blood (mean ± SD)	%CD8 ⁺		CD4 ⁺ /CD8 ⁺		%CD19 ⁺	
	MR	95% CI	MR	95% CI	MR	95% CI
1st tertile (14.09 ± 2.00)	1.00		1.00		1.00	
2nd tertile (18.56 ± 1.22)	0.82*	[0.68;0.97]	1.33*	[1.07;1.65]	0.98	[0.70;1.37]
3rd tertile (36.09 ± 38.45)	0.87	[0.72;1.05]	1.28*	[1.03;1.59]	0.71*	[0.51;0.99]

Values presented for each tertile are expressed in µg/L.

Parameter-specific actual confounders: %CD8⁺ – agricultural work; CD4⁺/CD8⁺ – red eyes when windy; %CD19⁺ – surgery in recent years, visits to the doctor for sickness, tea consumption, taking medicines, fish consumption > 2 times/week, water consumption, sickness in recent years.

* $P < 0.05$.

In our study high levels of Mn (2nd and 3rd tertile with mean concentrations of 18.56 and 36.09 µg/L, respectively) in blood decrease the MR of %CD8⁺ and %CD19⁺ and increased the MR of CD4⁺/CD8⁺ ratio. 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 in the groups with higher (mean concentration of 17 µg/L) and moderate (mean value of 11 µg/L) levels of Mn in blood.

Immunological biomarkers analysed in the present study appeared to be influenced by host factors, such as age and gender. The effect of age in the immune system has been described in several reports (Knight, 2000; Schindowski et al., 2002; Wick et al., 1997). Ageing 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 ratio. Similar results were published by several groups (Diamondstone et al., 1994; Frick et al., 2004; García-Lestón et al., 2012; Spencer et al., 2010). Increased neopterin concentrations and Kyn/Trp ratio 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., 2011a, 2012; Jentsch-Ullrich et al., 2005; Sansoni et al., 1993; Shahabuddin et al., 1998). The robust consistency of results from the Panasqueira study concerning the effect of ageing 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, with 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 have 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).

From all the clinical symptoms evaluated in the exposed populations, *cough with expectoration* and *taking medicines* significantly differ among study populations. 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.

5. Conclusions

Overall, the results of this study agree with those obtained in the preliminary studies (Coelho et al., 2011a, 2012), revealing immunotoxic effects experienced by populations environmentally and occupationally exposed to the metal(loid) contamination derived from the Panasqueira mining activities. The level of most exposure biomarkers was quantitatively associated with the intensity of immunotoxic effects. The immunological alterations observed may significantly increase the risk of developing immunological pathologies, and thus there is an urgent need for intervention in the area by the competent authorities to protect the health of populations working and living near the mine.

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Table 7
Logistic regression analysis for symptoms. Adjustment for age, smoking and symptom-specific actual confounders.

	Cough with expectoration		Alterations in skin		Alterations in smell		Alterations in taste		Red eyes when windy		Sick in recent years		Visits to doctor – sickness		Taking medicines	
	MR	95% CI	MR	95% CI	MR	95% CI	MR	95% CI	MR	95% CI	MR	95% CI	MR	95% CI	MR	95% CI
Controls (N = 40)	1.00		1.00		1.00		1.00		1.00		1.00		1.00		1.00	
Env. exposed (N = 41)	0.27*	[0.09;0.82]	0.69	[0.15;3.06]	0.37	[0.05;2.50]	0.48	[0.05;5.21]	1.75	[0.64;4.83]	0.97	[0.36;2.64]	1.27	[0.47;3.41]	0.52	[0.13;2.00]
Occup. exposed (N = 41)	0.31*	[0.10;0.93]	0.33	[0.05;2.05]	0.68	[0.15;3.08]	0.37	[0.04;3.68]	0.50	[0.19;1.34]	0.62	[0.23;1.72]	0.38	[0.14;1.06]	0.22*	[0.06;0.85]

Parameter-specific actual confounders: Cough with expectoration – surgery in recent years; Alterations in taste – use of pesticides in the last 3 years; Taking medicines – alcohol consumption, agricultural work. No parameter-specific actual confounder was identified for visits to the doctor for sickness, alterations in skin, alterations in smell, red eyes when windy, and sickness in recent years.

* $P < 0.05$.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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