Novel developmental immunotoxicology for monitoring the risk assessment for human populations from environmental pollution: alternative methods in vitro

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Abstract

There is increasing interest in the development and application of biomarkers for the purpose of risk assessment among human populations exposed to adverse environmental agents. This paper reports a way of monitoring the effects of air pollution on human health by using epidemiological data and in vitro immunotoxicological parameters in lead (Pb) environmentally exposed subjects. Epidemiological and human health statistics collected over the past few years indicate a negative impact of pollution resulting in the increase of the incidence of major diseases: it increases the occurrence of pulmonary diseases, asthma attacks, cardiovascular disease, heart attacks, the development of cancer, and mortality by these major diseases. A pilot study on the target subjects living in the highly polluted air (exposed group H. n=86) indicated statistically significant increased values of blood lead level (BLL) compared to subjects living in low or no Pb polluted air (control group C, n=37). Serum and hematological parameter values, some of them significantly different between the two human groups, also confirm the negative effects of air pollution. In an ex vivo study, using peripheral whole blood cultures and multiplexed immunoassay xMAP technology, the cytokine profiles in the exposed and unexposed subjects were tested, Th1 and pro-inflammatory cytokines TNF-α, IL-1β, IL-2, IFN-γ, IL-6, IL-8, and the regulatory Th2 interleukins IL-4, IL-10 were sensitively modulated in environmental exposure. The cytokine profile detection using small samples (500μg/L) of human whole blood is reproducible and can be effectively used as an in vitro biomarker in human epidemiological studies on environmentally exposed people.

Keywords: environmental, Pb exposure, health indicator, in vitro immunotoxicology, whole blood culture, cytokines, multiplexed immunoassay.

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1 Introduction

High levels of environmental pollution in developed countries are of particular interest because of the potential human health risk. Over the last decade, it was clearly established that air pollution is a possible public health hazard; evidence has been accumulated that suggests even short-term variations or low levels of air pollution are associated with measurable effects on mortality and morbidity [6, 18, 25, 27, 41, 43]. The pollutants can have serious consequences on human health, concluding that 24% of global disease is caused by environmental exposure and therefore could be avoided [49].

Evaluation of the potential health risks in Europe by sources, chemical properties and spatial distribution of cadmium (Cd), lead (Pb) and mercury (Hg) pollution, showed that up to one third of children in Europe have an "elevated" blood lead level (more than 10 μg/dL) and indicated that Pb poisoning is linked to neurodevelopmental problems and still threatens millions of children [50].

Pb is a hazardous, heavy metal that has a damaging impact on human health. Most of the Pb emitted in the air is expected to be associated with small particles, in sizes of 2.5 or 10 micrometers (PM 2.5μm; PM 10μm). Lead-containing dust is often removed from the air by rain and deposited on surface soil, where it may remain for many years. In addition, heavy rains may cause lead in surface soil to migrate into groundwater and eventually into water systems and food. Lead particles distribution in the environment as was notified in ATSDR, 1999 and 2005 [2], represents a health risk by their potential to penetrate and their long residence in the body [17, 20, 22, 27, 28, 44].

Bucharest is the biggest city in Romania, both as an urban area (238 sq Km) and population (about 2 million citizens resulting in a density of about 8107.6/sq Km). The political, economical and cultural capital, it is one of the most polluted cities in the country, because of the intense traffic, thermal power plants and industry [18].

A particularity of Bucharest city is its star shape with the centre applied on the city centre, representing the main crossway that connects the peripheral areas by main avenues and boulevards; this fact results in the highest level of pollution with NOx, CO, benzene, PM10, PM2.5 and Pb in the city centre, overloaded by the continuous development of car parks.

Air pollution has a long-term, detrimental impact on health, increasing the occurrence of death, asthma attacks, bronchitis, heart attacks, pulmonary and cardiovascular diseases, and cancer [4, 5, 11, 22, 25, 35].

There is scientific information on about 2% of the chemicals, but traditional toxicity testing in animal models often offer unreliable data to man.

Among the long-term goals of studying biological/biochemical responses to environmental contaminants is the identification of specific responses or biomarkers that might serve as early warning signals for ensuing pathologies in exposed organisms. In selecting appropriate biomarkers, certain key criteria need to be considered, including sensitivity, specificity, applicability, and reproducibility. For routine use and clinical implementation, biomarkers need to

be measurable in easily accessible body fluids such as serum, urine, saliva, cell or tissue culture supernatants [1, 16, 19, 39, 48].

New methods and tools developed in modern biology applied in various fields of toxicology allow earlier detection of toxic effects induced by chemical exposure on cellular and molecular level [1, 4, 9, 26, 33, 35, 39, 40]. Recently, there was a clear need of complementary research efforts using experimental in vitro and ex vivo methods to identify biomarkers plausibly associated with adverse effects of environmental pollution, additionally to epidemiological data [16, 26, 31, 47, 48].

A growing amount of evidence, and our previous experimental studies in vivo on Wistar rat indicate that high level of Pb causes toxic damage to the organs: liver and kidneys, heart, nervous and immune system, whereas even short-term exposure to low lead doses in vivo and in vitro has immunomodulatory effects that might reflect skewness of the immune response [6, 17, 19, 20, 22, 27, 29]. The existing epidemiologic data and data derived from the in vivo and in vitro studies support that the human immune system may be at increased risk following exposure to Pb and other heavy metals [3, 8, 15, 32, 37].

Few biological markers of immune function have been validated for use in epidemiological studies that involve delayed sample processing and analysis.

The cytokines release in whole blood cultures was indicated as a simple and relevant endpoint measurement in vitro because dysregulated cytokine production was observed in many immune mediated disorders and in chemical immunotoxicity [10, 19, 21, 23, 24, 34].

The detection of cytokine profile using the multiplexed immunoassay method, Luminex xMAP technology, is a practical and relevant assay allowing maximum flexibility to the end-user in creating the panel composition and selecting various cytokine families for simultaneous differential analysis in the major diseases associated with environmental exposure [14, 30, 36, 42].

This paper presents a way for monitoring the risk of environmental pollution on human health, using epidemiological data, usual biochemical parameters and in vitro immunotoxicological methods; the study was focused on Pb pollution and health effects.

2 Materials and methods

2.1 Epidemiological study design

Collecting information on air quality for 6 years (2000-2005), focused on PM10, PM2.5 and Pb level from the monitoring stations and from the periodic informative reports of Environmental Protection Agency-Bucharest (EPA-B), and statistical health indicators from The Center for Computing, Health Statistics and Medical Documentation (CCSMD), from Ministry of Health of Romania.

2.2 The pilot study

Selecting volunteers for two target human groups: subjects living in areas with high Pb exposure risk - group H (city centre of Bucharest), respectively, low or no Pb exposure risk, and control group C. The experimental protocol was

approved by the institutional ethical committee and the experiment was performed according to the Helsinki Declaration [12]. The subjects were checked by routine clinical chemistry - usual hematological and serum biochemical parameters performed on Horiba ABX and Hitachi 912 analyzers to establish their health status. Blood lead level (BLL) in the human subjects selected were tested by Atomic Absorption Spectroscopy (AAS) with electro-thermal atomization in furnace system.

2.2.1 Statistical analysis

Data are expressed as mean ±standard deviation, minim and maxim values. Student's *t test* was applied to compare the exposed group H with the control group C.

2.3 Experimental in vitro/ex vivo model

Whole blood culture was used for testing immunotoxicological parameters: venous blood collected from the target subjects, directly into vacutainer containing sodium heparin anticoagulant were diluted ¼ with RPMI 1640 containing fetal calf serum. Samples were distributed in 96 wells microplate, and stimulated with 10mg/L endotoxin LPS (Difco), and cultured 24-48h at 37^0C. Cell-free supernatants were collected and stored at -80^0C until analysis.

Cytokines release was evaluated by xMAP multiplexed immunoassay methods, based on fluorescent bead arrays (FBA) and performed on Luminex 200 system. The Luminex technology uses precise ratios of two fluorophores to create 100 different microspheres or bead sets. Each set is based on its internal dye ratios and can therefore carry a unique biological reagent. Antibodies are bound to the bead surface and serve as targets or as capture reagents for targets. We used a Fluorokine Map Multiplex Assays Kit (R&D Systems, USA). The Kit is configured as a Base Kit and a panel of Analyte Kits. Each Analyte Kit contains antibody-coated microplates and biotin-conjugated detection antibodies. The Base Kit contains all other reagents needed to perform the assay. Computer algorithms distinguish which assay is being carried on each bead quantifying the reaction based on fluorescent reporter signals. The cytokines panel investigated: IL-1β, TNF-α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-γ.

3 Results

From the information on air pollution in Bucharest during the 6 years (2000-2005) we have selected and analyzed particulate matter (PM) and Pb levels.

The data indicate that the traffic has a continuous increasing quota from the total Pb polluting level in the city centre. So, in 2005 the main contributors to the annual value of Pb pollution level, about 58836μg/m^3 air, were attributable: 56992 μg/m^3 air to the traffic, 1810μg/m^3 air to the industrial processes following a decreasing trend, and 90 μg/m^3 air to the thermal power plants.

Comparing the aggregated annual levels of PM10 pollution in Bucharest (2000-2005) shows a persisting high level, but with decreasing trend of the frequency of days exceeding maximum concentration level (MCL) (Table 1).

Table 1: Annual average concentration values for PM10 (μg/m^3 air).

Year	2000	2001	2002	2003	2004	2005
Mean PM10	0.140	0.147	0.135	0.134	0.141	0.138
MCL	0.208	0.185	0.175	0.175	0.167	0.161
% Days with level exceeding MCL	22.38	27.40	18.08	12.24	13.91	12.6

The annual data indicate the highest frequency of excessive levels of PM10 registered in 2001 and the lowest in 2003, keeping low levels in 2004 and 2005.

This decreasing trend for PM10 levels may be related to reducing the industrial battery production in the south-eastern area of Bucharest.

Pb content of PM, most of Pb emitted in the air, was performed by atomic absorbance mass spectrometry. Maximal Pb concentration admitted for daily averages is 0.7μg/m^3 of air. The annual mean of Pb levels in Bucharest showed a persisting high level, with about 68% of days in a year reach the levels over the EU limits in the close vicinity of industrial activity and in the city centre [46].

EPA (Environmental Protection Agency) proposes to revise the level of the standard to a level within the range of 0,10 to 0,30μg/m^3 in conjunction with retaining the current indicator of Pb in total suspended particles (Pb-TSP) but with allowance for the use of Pb-PM10 data, EPA also solicits comments on revising the indicator to Pb-PM10. This proposal is based on a thorough review of the latest scientific information on human health effects associated even with the low presence of Pb in the ambient air (EPA [17]).

Statistical health data available in The Information Bulletins on the public health of The Center for Computing, Health Statistics and Medical Documentation, (*CCSMD)* from Bucharest may indicate a detrimental impact of air pollution on health.

There is evidence that daily peak levels contributing to the year average can have significant impact on individuals' health. It increases occurrence indicators of morbidity and mortality by pulmonary diseases, asthma attacks, heart attacks, cardiovascular diseases, endocrine diseases and development of cancer. The exact impact of poor air quality on health is not thoroughly understood, but long term exposure to PM may increase susceptibility to infections, and those with chronic lung diseases or heart diseases may also have their conditions exacerbated.

The cardiovascular diseases have registered variable persisting high values of morbidity and mortality during the monitored period 2000-2005.

Morbidity by heart disease had reached spectacularly increased levels in 2004 and 2005 – about two times higher compared to 2001 and 2003 (data not shown).

What is of concern is the increased values for pulmonary diseases beginning with the year 2001 and persisting increasing trend in the next years, with highest level in 2005 (Table 2).

A positive aspect may be the stationary low values of the mortality by pulmonary diseases, contributing to amplifying values of the morbidity by more cases of remaining in evidence for the next year (Figure 1).

The mortality by asthma, after a high level reached in 2000, follows a decreasing trend in 2001, 2002 and 2003, while in 2004 and 2005 the level increased over those of 2000 year.

A great attention concerns the continuously increasing trend of morbidity by cancer in Bucharest quantified as the number of cases and as index on 100,000 citizens in the monitored period (Table 3).

Table 2: Bucharest quota of total morbidity and mortality by pulmonary diseases in Romania (2000–2006).

Health indicators (number of cases)		Years					
		2000	2001	2002	2003	2004	2005
Morbidity	**Total**	**151670**	**180428**	**180428**	**217819**	**236318**	**247311**
	Bucharest	*2600*	*15518*	*18353*	*19828*	*21671*	*23042*
Mortality	**Total**	**14824**	**14088**	**14058**	**14048**	**13678**	**13351**
	Bucharest	1099	1097	936	926	897	938

Table 3: Bucharest quota of total morbidity and mortality by cancer in Romania (2000–2006).

Health indicators **Total** (country) and in Bucharest		Years					
		2000	2001	2002	2003	2004	2005
Morbidity as Number of new cases	**Total**	**55027**	**57010**	**58751**	**58251**	**62789**	**61730**
	M. Bucharest	5575	6360	6072	5707	8408	9540
Morbidity as Index/100,000 citizens	**Total**	**245.3**	**254.4**	**269.6**	**268.0**	**289.7**	**285.5**
	M. Bucharest	277.5	318.5	313.9	259.8	436.2	495.6
Mortality Number of cases	**Total**	**41290**	**42750**	**43191**	**43676**	**43985**	**44906**
	M. Bucharest	4804	4880	5032	5009	4918	5137
Mortality Index/100,000 citizens	**Total**	**184.02**	**190.78**	**198.17**	**201.0**	**202.9**	**202.9**
	M. Bucharest	243.07	247.44	262.21	261.3	256.8	256.8

The annual values of the mortality by cancer remaining at about a constant level may be taken as a measure of surviving period of patients, remaining in evidence (Figure 2).

The statistical data indicate a concerning spectacularly rate of increasing trend of cancer incidence with the highest values for 2004 and 2005 years.

Blood lead level performed on the two groups of volunteers: group H, subjects living in the Bucharest's high polluted area and the control group C, subjects living in low or no polluted areas (villages and small cities).

The BLL levels of the group H (n=36) were 11.539 ± 4.688μg/dL, ranging between 4.00 - 21.93μg/dL, whereas in the case of the group C (n=20), were 3.117 ± 1.950μg/dL (mean ± SD), with values from 0.808 - 8.58 μ/dL.

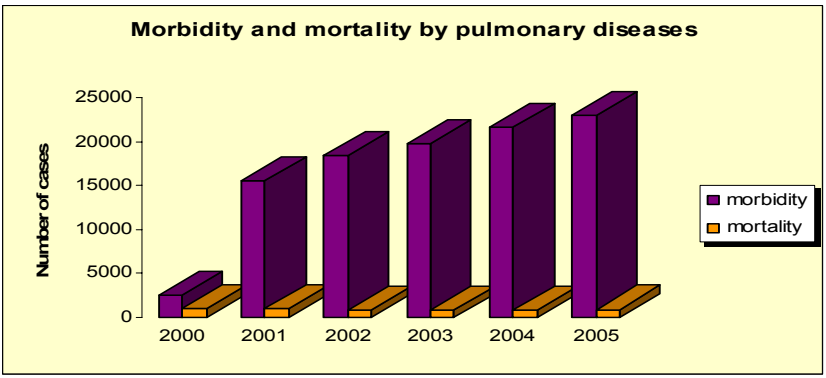

Figure 1: Morbidity and mortality by pulmonary diseases in Bucharest.

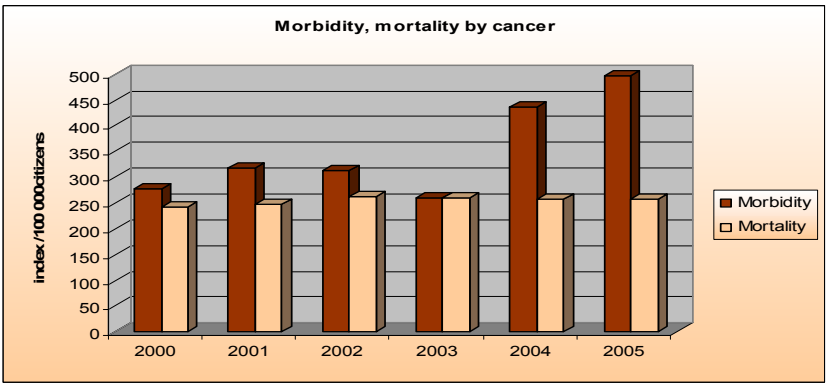

Figure 2: Morbidity and mortality by cancer in Bucharest.

Statistical analysis was made by *student t-test*, showing that the BLL level was significantly increased ($p<0.001$) in group H compared to the control group C.

Biological parameters are presented in Tables 4 and 5.

Values of the hematological parameters white blood cells (WBC), red blood cells (RBC), platelet (PLT), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV)*;* mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC), indicate variability in both groups, remaining in, or close to biological ranges. Some of these parameters are presented in Table 4.

From biochemical serum parameters, the serum levels of creatinine, glycemia, cholesterol, AST, indicators of the renal and hepatic functions showed statistically significant differences ($p<0.05$) in the exposed group compared to control, indicating disturbances in metabolic activities in these target organs of

Table 4: Levels of the hematological parameters; comparative analysis.

Groups	WBC (10^3/mm^3)	RBC (10^6/mm^3)	Hb (g/dL)	MCHC (g/dL)	PLT (10^3/mm^3)
group C n=12	6.00± 1.55* (3.7 – 8.6)	4.57 ± 0.30 (4.16- 5.15)	12.72± 1.58 (9.5– 15.4)	31.78± 1.46* (28.7 – 33.2)	331.83± 35.75 (232 -627)
group H n=29	7.05 ± 1.99 (4.5-10.9)	4.46 ± 0.44 (3.58-5.66)	11.92 ±1.53 (9.6 - 15)	30.90± 1.25 (28.5 – 33.4)	265 ± 60.83 (156-405)

C, control group; H, exposed group; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration, PLT, platelets. ***p<0.05*** *statistically significant differences between the groups.*

Table 5: Levels of some serum biochemical parameters.

Group	Creatinine (mg/dl)	Glycemia (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	ALT (U/L)	AST (U/L)
C (n=37)	1.03 ± 0.24 (0.5-0.73)	93.93 ± 12.58 (74-132)	189.61 ±33.16 (143-266)	58.45 ±15.22 (34.1-81.5)	22.66 ±14.76 (5.9-64.6)	19.46 ±7.06 (8.8-33.34)
H (n=86)	0.95 ±0.20* (0.45-1.59)	100.18 ±18.66* (71-204)	201.63 ±43.37* (144-370)	59.89 ±23.36 (26.3-211)	25.84 ± 17.07 (6.7-95.2)	26.10 ±11.56 * (11.2-56.5)

C, control group; H, exposed group; HDL, low density lipoproteins; ALT, alanine aminotransferase; AST, aspartate aminotransferase. ***p<0.05*** *statistically significant differences between the groups.*

toxic aggression. The differences in the exposed compared to the unexposed group cannot be exclusively associated with environmental exposure because some subjects from the unexposed group show similar types of behavior.

Cytokine release evaluated by xMAP technology in the *ex vivo* system: whole blood samples were collected from subjects of control group C having stimulated low BLL, and of exposed group H, with high BLL and cultured 48 hours. Cytokine levels were quantified in the culture supernatants. The results showed that environmental lead exposure affected secretion of pro-inflammatory cytokines: tumor necrosis factor (TNF)-α, and interleukin IL-1ß, as well as Th1 cytokines - interferon (IFN)-γ, IL-8, IL-6 were reduced, while IL-2 and IL-12 were increased in exposed group H as compared to control group C (Figure 3).

Th2 cytokines IL-4 and IL-10 were higher in exposed group compared to control, with unexpected high values for IL-10 (Figure 4).

4 Discussion

The negative impacts of lead pollution on human health are well documented. It is regarded to be one of the most serious health problems facing populations,

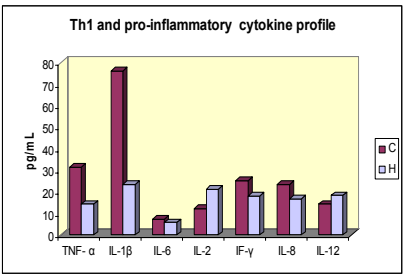

Figure 3: Th1 cytokine profiles.

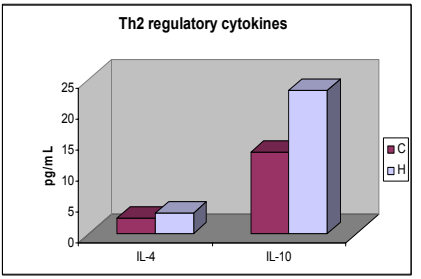

Figure 4: Levels of the Th2 cytokines.

particularly children. Common symptoms include IQ loss, reading and learning difficulties, hearing loss, difficulties in concentration, adverse effects on kidney function, blood chemistry, and the cardiovascular system as well as adverse reproductive effects for women. Pb has long been known to alter the hematological system by inhibiting the activities of several enzymes involved in heme biosynthesis, particularly d-aminolevulinic acid dehydratase (ALAD), beginning at Pb blood level <10μg/dL, according to ATSDR -2005 [2].

The key indicator for biomonitoring lead exposure is the blood lead level (BLL). Given the widespread distribution of lead in the environment, everyone has a low background level. Recent studies have indicated that, depending on the individual sensitivity to lead-induced neurotoxicity and individual susceptibility, even a BLL below 10μg/dl can lead to negative neurobehavioral effects in children [6].

There are data indicating that increasing trend or persisting high level of air pollution is positively correlated with the morbidity and mortality by major diseases.

Our results indicate that the group of subjects living (about 8-10 years) in areas with a high level of Pb polluting air present a correlated trend of BLL, with a significantly increased level ($p<0.001$) in H group compared to the control C group.

This may induce cellular and tissue damages mediated by reactive oxygen species (ROS) by developing oxidative stress [7] and the associated events-genotoxic [27], apoptotic effects [8, 9, 12, 29]. Effects of Pb on immune response have been reported by several investigators, but no clear picture emerges from these data regarding the potential immunotoxic effects. New developmental immunological biomarkers indicated that modulation of T helper (Th) cell response is one means by which xenobiotics may cause immunotoxicity. In light of previous investigations it seems that for the most of the major diseases, including cancer, the approach in order to reveal the cellular interactions involved would be to measure cytokine profile changes [10, 21, 23, 24, 30, 31, 34, 36, 38].

The pulmonary diseases are associated with chronic bronchitis, fibrosis and tissue damage, oxidative stress and inflammatory response [4, 10]. Increased

levels of pro-inflammatory cytokines IL-6, IL-1β, TNF-ά and IL-8, and of the anti-inflammatory cytokine IL-10 were observed in pulmonary diseases. Asthma process is driven and maintained by a subset of chronically activated T cells, sensitized against allergenic antigens which "home" to the lung after exposure. In general, allergens induce Th2 cell response, which produces interleukin IL-4, an essential cofactor for IgE production [14, 31].

Our results obtained by ex vivo whole blood culture system, in accord with existing data of in vivo and in vitro studies, indicate that Pb might modulate the production of Th factors, altering preferentially Th2 cells, suggested here by the increased IL-4 and IL-10 overproduction. It was shown that Th1 cytokines were decreased and Th2 activation was enhanced by environmental Pb, suggesting the immunosuppressive effects for this level of exposure. The lower Th1/Th2 ratio that resulted confirms some previous reports about immunomodulation by Pb [28, 32, 33]. High doses of Pb (occupational long term exposure) may induce over immunoreactivity with allergic and autoimmune reactions. This differential effect of Pb may explain, at least in part, the reported diversity of in vivo effects on immune response [3, 6, 13, 20, 22, 24, 27, 28, 29, 44]. Th2 reactions are driven by IL-4, IL-5, IL-10, and IL-13 and result in an increase in IgE production. Indeed, significantly elevated levels of IgE have been found in lead-exposed workers, although no significant relationship between blood lead levels and serum IL-4 or IFN-γ levels was observed [24]. The elevated production of IL-4 and/or IL-10 can induce and maintain a Th2 immune response and might contribute to increased susceptibility to pathologic agents as well as to the incidence of allergic hypersensitivity and/or Th2-dominated autoimmune diseases.

5 Conclusions

Environmental air pollution data and human health statistics collected over the past few years indicate a negative impact of pollution resulting in increase of the incidence of major diseases in our country and especially in Bucharest and in other big cities.

Biomonitoring of environmental chemicals in the human body, specifically in blood, urine, serum, saliva, or tissues, represents the usual mean of evaluating the toxic effective dose (BLL) and biological impact of exposure on biochemical parameters levels.

Results from the present study consistent with the experimental studies, indicate correlation between Pb exposure and immunological competence.

In vitro/ ex vivo novel developmental immunotoxicology by multiplexed immunoassay methods may represent a novel candidate for biomarkers in environmental risk assessment, allowing detection of earlier effects of the toxics on cellular level.

Trends of air pollution indicators and health risk outcomes represent necessary information to citizens because people should not only know about the level of pollution but should also avoid exposure to Pb and other noxes.

Acknowledgement

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