

Assessment of metal contaminants in non-small cell lung cancer by EDX microanalysis

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Abstract

Human cardio-respiratory diseases are strongly correlated to concentrations of atmospheric elements. Bioaccumulation of heavy metals is strictly monitored, because of its possible toxic effects. In this work, we utilized the EDX microanalysis in order to identify the potential heavy metal accumulation in the lung tissue. To this aim, we enrolled 45 human lung biopsies: 15 non-small cell lung cancers, 15 lung benign lesions and 15 control biopsies. Lung samples were both paraffin embedded for light microscopy study and epon-epoxid embedded for transmission electron microscopy. EDX microanalysis was performed on 100 nm thick unstained ultrathin-sections placed on specific copper grids. Our results demonstrated that the EDX technology was particularly efficient in the study of elemental composition of lung tissues, where we found heavy metals, such as Cobalt (Co), Chromium (Cr), Manganese (Mn) and Lead (Pb). Furthermore, in malignant lesions we demonstrated the presence of multiple bio-accumulated elements. In fact, a high rate of lung cancers was associated with the presence of 3 or more bio-accumulated elements compared to benign lesions and control tissue (91.7%, 0%, 8.3%, respectively). The environmental impact on pulmonary carcinogenesis could be better clarified by demonstrating the presence of polluting agents in lung tissues. The application of EDX microanalysis on biological tissues could shed new light in the study of the possible bioaccumulation of polluting agents in different human organs and systems.

Introduction

Active cigarettes smoking, occupational exposures, residential radon, and environmental

tobacco smoke are risk factors for lung cancer, which is the most common cause of cancer death worldwide.¹ Air pollutants, especially particulate substances, such as polycyclic aromatic hydrocarbons and other genotoxic chemicals, are suspected to increase the risk of lung cancer.^{2,3}

In the large urban centers of many industrialized countries, a strong correlation has been identified between mortality caused by cardio-respiratory diseases and atmospheric concentrations of particulate matter (from particles below 10 μm , called PM 10, to smaller ones, the PM 2.5).^{4,5} In the last few years, numerous studies postulated the synergistic effects of oxidative stress and inflammation, as being the major biochemical pathways of PM-induced toxicity and health effects.^{6,7} The depth of penetration and deposition of PM depends on the particle size, shape and density.⁸ Beside these physical parameters, PM-induced toxicity is also affected by its chemical composition and source.⁹ Among the elements which constitute PM 10 and PM 2.5, heavy metals share possible toxic effects induced by their bio-accumulation. *In vitro* experimental data suggest heavy metal toxicity and that the onset of metal poisoning could be accompanied by recognizable acute symptoms.¹⁰

Cobalt and its compounds induced aneuploidies, micronuclei and chromosomal aberrations in the bone marrow in rodents after intra-tracheal infusion¹¹ and mutations of the K-ras.¹² The exposure to chromium (VI) compounds is consistently associated with the incidence of lung cancers both in experimental models and in humans.¹³ Lead exposure can occur by inhalation or ingestion. These substances are distributed throughout the body *via* the blood stream. The toxic nature of lead is well-documented and is known to affect all organs and functions.¹⁴ Manganese, an essential element for human health in trace amounts, is toxic at high doses. Since manganese is globally replacing lead in gasoline, an evaluation of its potential cancer effects is relevant.¹⁵

Despite this data, the long-term effects of bioaccumulation of heavy metals and their involvement in human chronic diseases are still controversial.

The aim of this work is to investigate the presence of toxic elements, such as Co, Cr, Mn, and Pb in the lung tissue explored by EDX microanalysis. This data could enable us to correlate the presence of these elements in the lung tissues with pulmonary diseases.

Materials and Methods

Histological analysis

In this retrospective study, we selected 45

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Contributions: MS, AO, EB, conceived, carried out experiments and analyzed data; IT, SB, carried out experiments and analyzed data; MS and AO equally contributed to the work. All authors were involved in writing the paper and gave final approval of the submitted and published versions.

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consecutive human lung biopsies from our archive (Table 1). All biopsies were fixed with 10% buffer formalin and paraffin embedded; 4- μm -thick sections were stained with hematoxylin and eosin (H&E).¹⁶ Two pathologists blindly classified the tissues.

Immunohistochemistry

The phenotype of neoplasia was characterized by the presence of cytokeratin 5/6 and p63 (typically expressed by squamous cell carcinomas) and by the thyroid transcription factor 1 (TTF-1) and cytokeratin 7 (typically expressed by adenocarcinomas). Immunohistochemistry was performed by Bench Mark automatized system (Ventana, Tucson, AZ, USA). Briefly, 4- μm -thick sections were pre-treated with CC1 reagent (Ventana) for 30 min at 95°C and then incubated respectively with mouse monoclonal anti-Cytokeratin 5/6 for 30 min (pre-diluted clone D5/16B4, Ventana), rabbit monoclonal anti-Cytokeratin 7 for 30 min (pre-diluted clone SP52, Ventana), mouse monoclonal anti-p63 for 40 min (pre-diluted clone 4A4,

Ventana) and rabbit monoclonal anti-TTF-1 for 30 min (pre-diluted clone SP141, Ventana). Washings were performed with reaction buffers produced by Ventana; reactions were revealed by ultraView Universal DAB Detection Kit (Ventana).

Ultrastructural analysis

Lung samples were 4% paraformaldehyde fixed, 2% osmium tetroxide post-fixed and embedded in epon resin as previously reported.¹⁷ After washing 0.1 μ M with a phosphate buffer, tissues were dehydrated by 30%, 50%, 70%, 95% and absolute ethanol. After the short propylene oxide incubation, samples were embedded in epon epoxy resin (Agar Scientific, Stansted Essex CM24 8GF United Kingdom).¹⁸ Eponepoxy resin embedded tissues were sectioned¹⁸ and stained with uranyl acetate and lead citrate as previously described.¹⁹

All samples were examined by a transmission electron microscope Hitachi H-7100 FA (Hitachi, Schaumburg, IL, USA).

EDX microanalysis

The EDX microanalysis is a technology that performs the elemental and chemical analysis of a sample in transmission electron microscopy. When the electron beam in an electron microscope hits a thin sample, some atoms of the sample will be excited or ionized. When they return to their ground state, they will emit characteristic x-rays. The x-ray emission at different wavelengths may then be measured by a photon-energy-sensitive detector. The EDX detector system performs a simultaneous display of all mid-energy (1-20 keV) x-rays collected during any individual analysis period. Therefore it is possible to detect those elements with AN>10. The minimal detectable elemental concentration, which requires some signal averaging, is approximately 0.1 mmol per kg of dry specimen (*i.e.*, 10 ppm), whereas spatial resolution ranges from about 10 nm to a few micrometers. For the EDX microanalysis 100 nm-thick unstained ultrathin sections were placed on specific copper grids. The EDX spectra were acquired by a Hitachi 7100FA transmission electron microscope and an EDX detector (Thermo Scientific) at an acceleration voltage of 75 KeV and 12000 magnification. Spectra were semi-quantitatively analyzed by the Noram System Six software (Thermo Scientific, Waltham, MA USA) using the standardless Cliff-Lorimer k-factor method.²⁰ The EDX microanalysis system was calibrated using the x-ray microanalysis standard (Micro-Analysis Consultants Ltd, Cambridgeshire UK). The EDX system was set up to detect Co, Cr, Mn and Pb. Water pollutant elements like As were excluded from the analysis, since they are contaminants of the

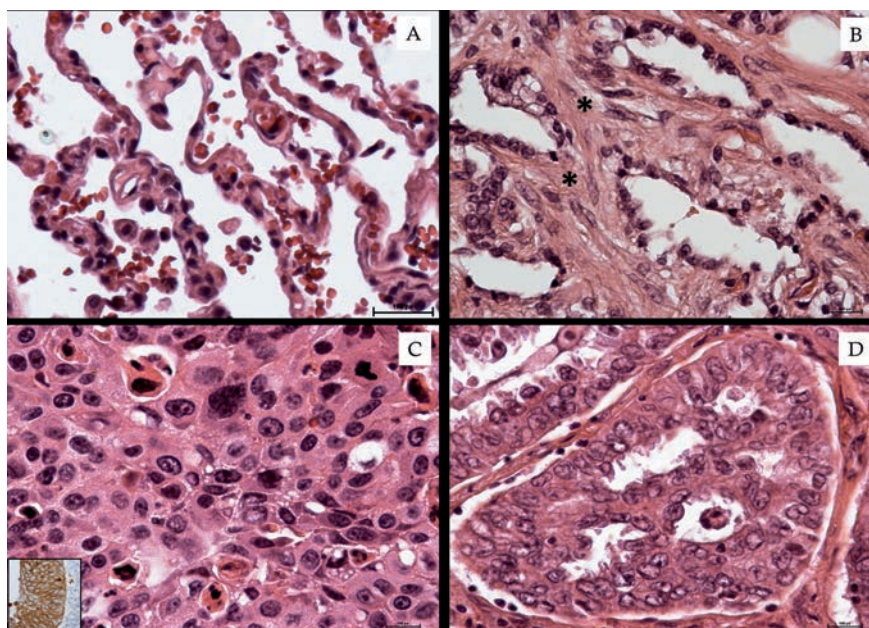


Figure 1. Lung tissue morphological study. A) Normal lung tissue characterized by alveoli with thin walls. B) Pulmonary fibrosis; asterisks indicate collagen deposition in the alveolar septa. C) Squamous cell carcinoma (inset shows Cytokeratin 5/6 positivity). D) Well differentiated adenocarcinoma.

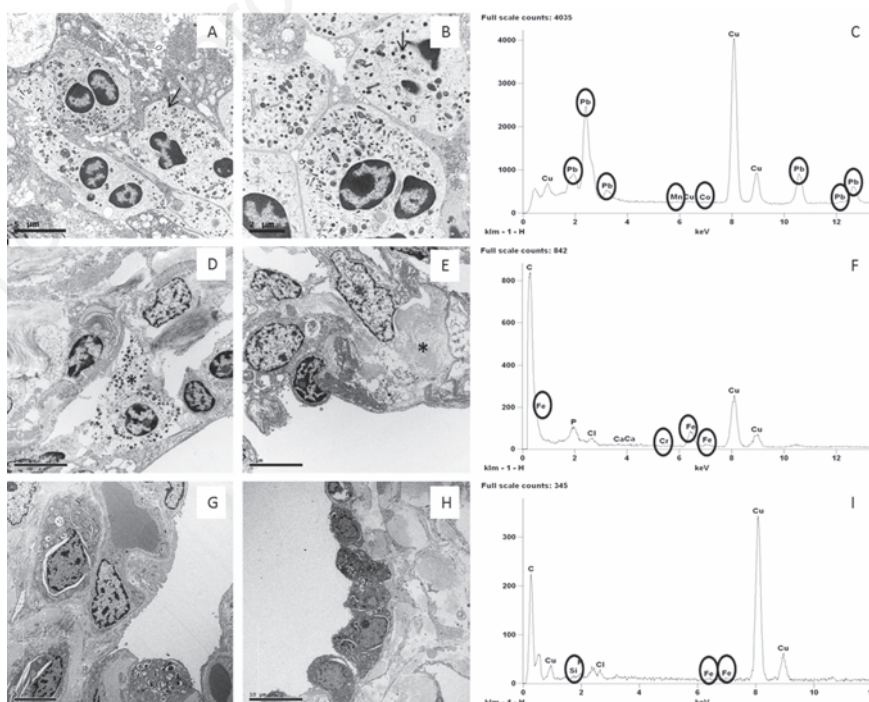


Figure 2. Ultrastructural and EDX analysis of lung tissues. A,B) Lung adenocarcinoma cells with dense granules 400 to 600 nm in diameter (arrow) and large nucleoli. C) Toxic elements detected in one case of NSCLC. D) Inflammatory infiltrates (asterisk indicate a mastocyte) in interstitium of BL tissue. E) Chronic interstitial pneumonia; note lung cells and collagen deposition (asterisk). F) Toxic elements detected in one case of benign lesion. G,H) Lung tissues without significant pulmonary pathology. I) Toxic elements detected in one case of control tissues.

buffer solutions used during histological sample preparation.

The sample standards used as controls were: Dural (Al, Cu, Mg), Apatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$), Lead Sulphide (PbS), Chromite (FeCr_2O_4), Chromium (Cr), Iron (Fe), Manganese (Mn), Silicon (Si), Tungsten (W), Zinc (Zn), Cadmium sulfide (CdS) and Cobalt (Co). For each sample, the microanalysis acquisition was performed in 15 fields selected randomly.

Animal model

To assess the ability of the EDX microanalysis to detect the accumulation of inhaled metals into the biological tissues, 4 mice, C57 black strain, were exposed to SiO_2 particles. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and carried out according to the Italian and European rules (Leg. Decree 116/92; EC 609/86; European Directive 2010/63/EU). Mice were exposed to SiO_2 dusts for periods of 15, 30, and 50 days in chambers equipped with conical-type dust generators. The animals were exposed to the dusts for 6 h a day, 3 days a week.

Statistical analysis

Descriptive statistics was used to summarize pertinent study information. Categorical variables were reported as frequencies and percentage values, whereas continuous variables were summarized by mean values and their relative standard deviations.

Correlations between categorical variables were evaluated through non-parametric tests, such as the Pearson's Chi-Square test or the Fisher exact test, when appropriate. P-values <0.05 denote statistically significant correlations. Statistical analyses were carried out using the SPSS software (SPSS ver. 21.0, SPSS Inc., Chicago, IL, USA).

Results

Morphological analysis

The study of H&E sections, immunostaining reactions and ultrastructural characterizations allowed us to classify human lung biopsies according to WHO 2012 as follows: 15 non-small cell lung cancers (NSCLC) classified as adeno-carcinomas (9 cases) and squamous cell carcinomas (6 cases), 15 benign lung lesions (BL) classified as chronic interstitial pneumonia (8 cases), idiopathic pulmonary fibrosis (5 cases) and adipose fibrosis (2 cases), and 15 lung tissue from patients negative for pulmonary pathology (Controls) (Figures 1 and 2).

NSCLC cases included 10 male patients and 5 female patients (mean age 60.13 ± 2.54

years), among them 12 were smokers. The BL group was composed of 12 male and 3 female patients (mean age 66.53 ± 2.05 years), among them 10 were smokers. Control group consisted of 8 male and 7 female patients (mean age 66.14 ± 1.77 years), 10 among them were smokers (Table 1). Mouse lung tissues showed fibrotic and inflammatory reactions (*not shown*).

Elemental analysis

Ultrastructural microanalysis was set up in order to characterize the presence of heavy metal in lung tissues. The demonstration of Si particles in lung tissues from mice after metal fume inhalation proved the reliability of the EDX assay (Figure 3). In the human lung specimens the EDX microanalysis showed a great variability in terms of distributions among the three groups of tissues. Lung cancer was characterized by a high presence of

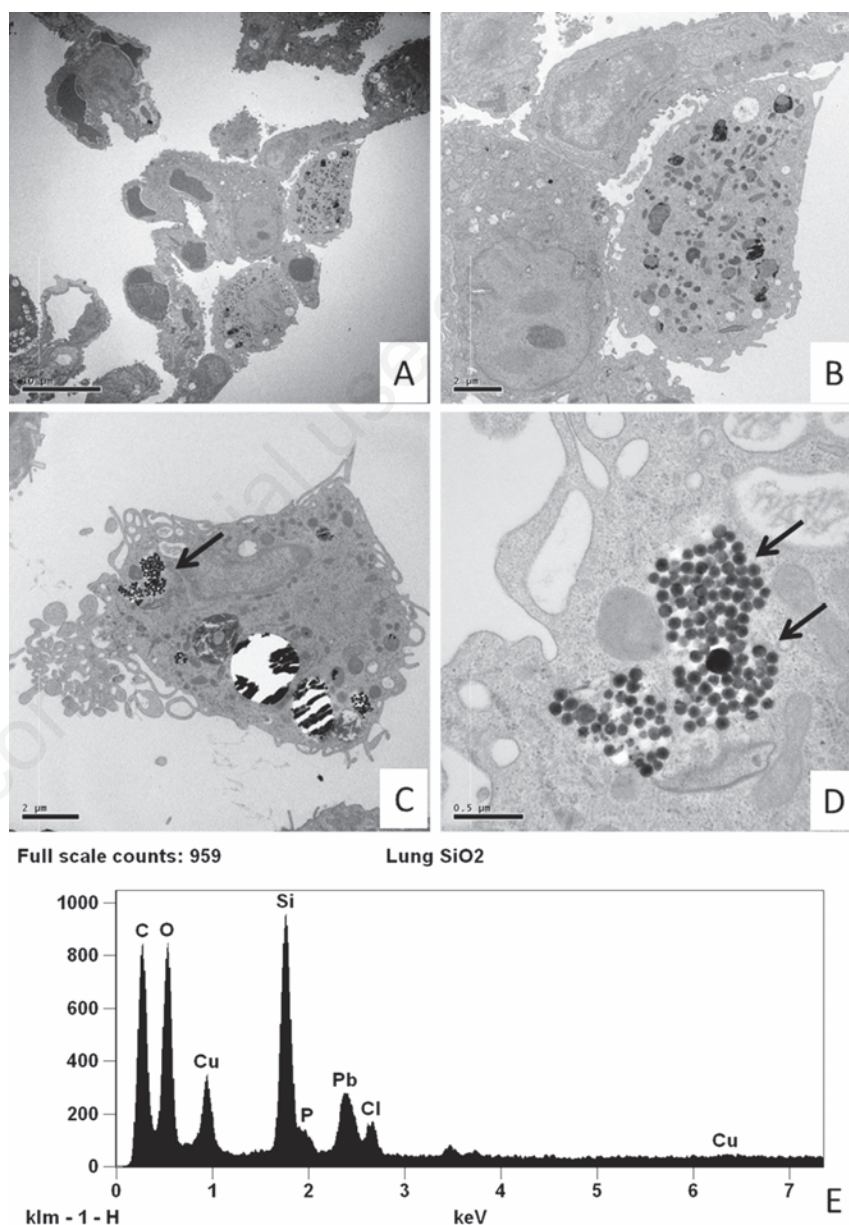


Figure 3. Transmission electron microscopy and EDX microanalysis of murine lung tissues. A) Low magnification of thin walled alveoli (15 day of SiO_2 exposure). B) Histiocyte with a moderate amount of electron dense granules. C,D) Mice lung electron micrographs after 50 day of SiO_2 exposure show an elevated amount of small particles (arrows) into the cytoplasm of histiocytes. E) EDX spectrum shows that the particles indicated in the panel D are made of Si. Other elements can be referred to the composition of the support grid (Cu), staining solution (Pb) and biological tissue (Cl, C, O and P).

heavy metal particles. At least one of these elements was detected in each NSCLC. However a non-statistically significant difference between adenocarcinomas and squamous cell carcinomas was observed (*data not shown*). On the contrary, the frequency of accumulation of toxic elements both in BL and in the control group was less and the presence of two or more elements was only occasionally reported. As for the individual elements, Pb was detected in 86.7% of NSCLC samples, whereas Cr and Co were present in 80% and 73.3%, respectively. Differences among the three groups for these three elements were statistically significant (Figure 4 A,B,C). As to Mn, the different presence of this element in the three groups was not significant, although the NSCLC tissues still showed higher percentage values (Figure 4D).

A high rate of NSCLC biopsies showed the co-presence of more than one bio-accumulated element. Interestingly, we could find significant differences ($P < 0.0001$) among the groups, when we compared the bio-accumulation of two or more elements. In particular, at least 2 elements were present in 100% of NSCLC, 40% of BL and 20% of controls (Figure 5A), whereas more than 3 elements were found in 73.3% of NSCLC and 8.3% of controls. No BL showed a combination of more than 3 heavy metals (Figure 5B).

Discussion

Our results demonstrated that the EDX microanalysis is a reliable method to reveal inhaled metal fumes.

The most significant elements accumulated in human lung tissues were Pb, Cr, Co and Mn. It should be noted in particular that these heavy metals coincided with those emitted in the atmosphere by natural and/or anthropic sources.²¹⁻²³ The most frequent element in NSCLC and BL tissue was Pb (Figure 4A). The toxicity of Pb and its compounds is well-known and is prominently associated with anemia and developmental disorders. Genotoxic and cancerogenic effects of lead compounds are well-documented in *in vitro* systems, experimental animal models and in lead exposure studies in humans.¹⁴ Co and Cr were more frequently found in NSCLC (Figure 4 B,C). Exposure to Cr and Co compounds has been consistently associated with incidences of lung cancers in humans and experimental animals.²⁴ Cells of workers exposed to dusts of chromium compounds showed elevated frequencies of DNA strand breaks,²⁵ sister-chromatid exchanges and micronuclei.²⁶ Inorganic cobalt compounds, both soluble and particulates, caused lung tumors in animal experiments, whereas the

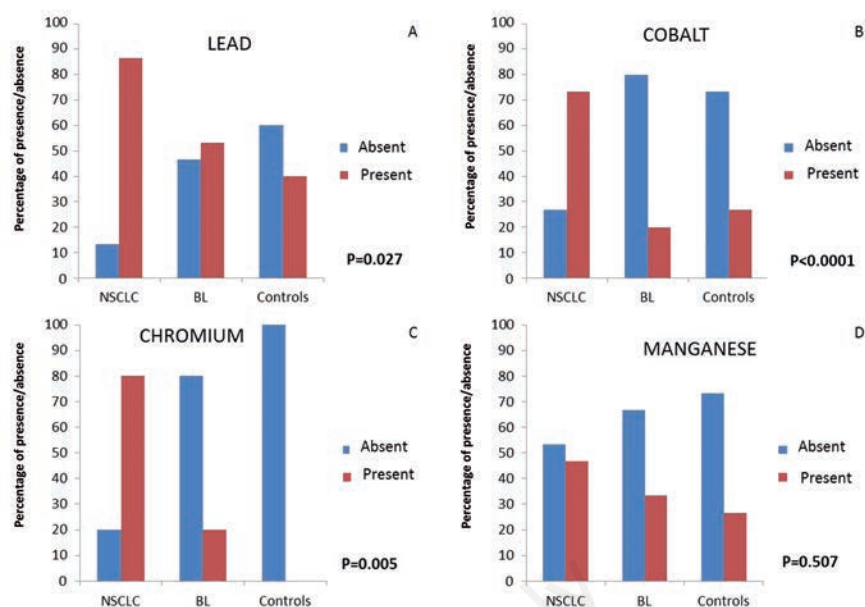


Figure 4. Elemental analysis. Percentage distributions of the four heavy metal according to the three group of tissues: A) lead (Pb); B) cobalt (Co); C) chromium (Cr); D) manganese (Mn). The presence (red column) and absence (blue column) of heavy metal revealed respectively in Non Small Cell Lung Cancer (NSCLC), benign lesions (BL) and control cases (Controls) are reported as percentage. *Chi-Square's test. P-values refer to comparisons among the three groups of tissues according to the presence of each specific element.

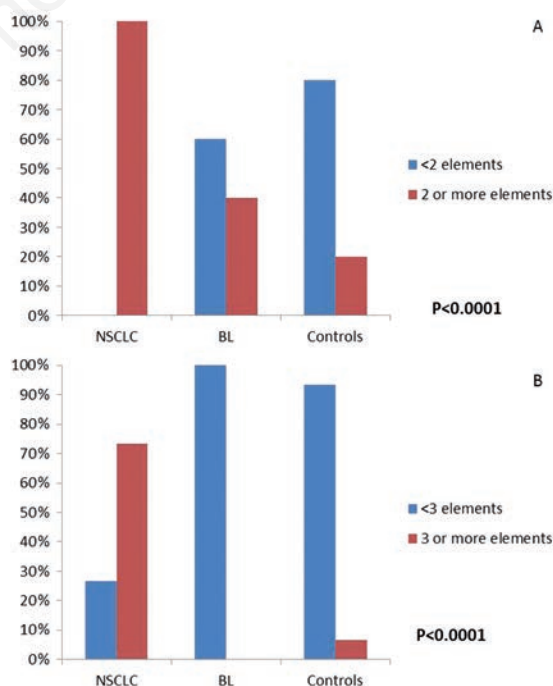


Figure 5. Percentage distribution of the number of heavy metals according to the three groups of tissues: Non Small Cell Lung Cancer (NSCLC), benign lesions (BL) and control cases (Controls). *Chi-Square's test. P-values refer to comparisons among the three groups of tissues according to the simultaneous presence of elements: 2 or more elements, red column; less than 2, blue columns.

epidemiological findings of the increased lung cancer incidence of cobalt-exposed workers are not regarded as being conclusive, because of the co-exposure to other carcinogenic sub-

stances.²⁷ In an inhalation carcinogenicity study with cobalt sulfate, mutations in the K-ras oncogene were observed in lung tumor tissues of exposed mice.¹² Moreover, in industrial-

ized countries, car exhaust fumes and cigarette smoke, which are the main sources of pollution impacting on human health, contain traces of all the above-mentioned elements.²⁸ Manganese is a transition metal necessary for life processes in trace amounts, but it is toxic at higher doses.¹⁵ The presence of Mn in NSCLC and BL tissues led us to postulate a relationship with the pathological status (Figure 4D). When Mn particles are present in the atmosphere, their little size (≤ 10 nm) allows them to easily penetrate into the bronchiole mucosa. Hence, an inflammatory process can start and lead to epithelial inflammation, interstitial pneumonia and granulomatous reactions.²⁹ In fact, although it was typically considered a neurotoxin,³⁰ excessive Mn has also been associated to increased susceptibility to pulmonary infection,³¹ pulmonary neoplasia,³² infant mortality,³³ and criminal and violent behaviors.³⁴

The progressive environmental pollution and the diffusion of inappropriate lifestyles have significantly contributed to the increase in the incidence of lung lesions. Our results show significant differences in the bioaccumulation of toxic elements, such as Co, Cr and Pb, between pulmonary cancer lesions (NSCLC) and benign lesions. The *ex vivo* identification of these toxic elements in the lung tissue could better clarify its environmental impact on lung carcinogenesis. To this aim we used the EDX microanalysis to associate morphological / ultra-structural data with the bio-accumulation of toxic elements related to air pollution. Despite this technology may be difficult to set up and use, it proved particularly efficient for the study of lung tissues.

Lastly, the presence of heavy metals in the examined tissues leaves a fundamental question unanswered: should the bio-accumulation of air pollutant be considered as a primary insult related to tumorigenesis or rather is it due to intrinsic characteristics of the neoplastic tissues which, for unknown reasons, tend to behave as a storage tissue of the elements? As to future prospective research, it would be interesting to verify, through an EDX microanalysis, the possible bio-accumulation of these pollutants and toxic elements in different human organs and systems.

Table 1. Patients characteristics and EDX microanalysis.

Lesion	Age	Smoke habit	Elements
Adeno-carcinoma	71	Yes	Co; Cr; Pb
Adeno-carcinoma	58	Yes	Co; Cr; Pb
Adeno-carcinoma	62	No	Co; Cr; Mn
Adeno-carcinoma	56	Yes	Co; Cr; Pb
Adeno-carcinoma	59	Yes	Co; Cr; Mn; Pb
Adeno-carcinoma	58	Yes	Co; Cr; Pb
Adeno-carcinoma	49	Yes	Mn; Pb
Adeno-carcinoma	68	Yes	Co; Cr; Pb
Adeno-carcinoma	62	Yes	Co; Cr; Mn; Pb
Squamous cell carcinoma	57	Yes	Cr; Pb
Squamous cell carcinoma	53	Yes	Co; Cr
Squamous cell carcinoma	66	Yes	Mn; Pb
Squamous cell carcinoma	58	Yes	Co; Mn; Pb
Squamous cell carcinoma	64	Yes	Cr; Pb
Squamous cell carcinoma	61	No	Co; Cr; Mn
Chronic interstitial pneumonia	57	Yes	Cr; Mn
Chronic interstitial pneumonia	67	Yes	Co
Chronic interstitial pneumonia	82	Yes	Pb
Chronic interstitial pneumonia	58	Yes	Pb
Chronic interstitial pneumonia	49	No	Mn
Chronic interstitial pneumonia	67	Yes	Mn; Pb
Chronic interstitial pneumonia	77	No	-
Chronic interstitial pneumonia	64	Yes	Pb
Idiopathic pulmonary fibrosis	69	Yes	Mn; Pb
Idiopathic pulmonary fibrosis	77	No	Co
Idiopathic pulmonary fibrosis	72	Yes	Mn; Pb
Idiopathic pulmonary fibrosis	60	No	-
Idiopathic pulmonary fibrosis	64	Yes	Cr; Pb
Adipose fibrosis	59	Yes	Co; Cr
Adipose fibrosis	61	Yes	Pb
Normal lung tissue	68	Yes	Mn
Normal lung tissue	67	Yes	Co; Mn; Mn
Normal lung tissue	75	No	-
Normal lung tissue	74	No	-
Normal lung tissue	54	Yes	Pb
Normal lung tissue	58	No	-
Normal lung tissue	65	Yes	Pb
Normal lung tissue	57	No	-
Normal lung tissue	68	Yes	Mn; Pb
Normal lung tissue	70	No	Co
Normal lung tissue	71	Yes	Mn
Normal lung tissue	58	No	-
Normal lung tissue	69	Yes	Co
Normal lung tissue	72	Yes	Pb
Normal lung tissue	51	Yes	Pb

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