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Evaluation of essential and toxic metal imbalances in the blood of various types of lung cancer patients

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ABSTRACT

Lung cancer is a major cause of the cancer-related deaths worldwide and exposure to trace metals is a well-documented risk factor for it. Present study is carried out to evaluate the comparative distribution, correlation and multivariate apportionment of selected essential and toxic metals (Fe, Zn, Cu, Sr, Li, Co, Mn, Ni, Cr, Cd and Pb) in the blood of lung cancer patients and healthy donors/controls. The blood metal levels were measured using flame atomic absorption spectrophotometry employing mineral acid digestion method. The average concentrations of Fe, Li, Ni and Cd were significantly higher ($p < 0.05$) in the blood of patients than controls; however, Sr, Co, Mn and Cr were significantly elevated for the controls. Significant/strong correlations were found between Pb-Cr, Cr-Li, Pb-Co, Cd-Cr and Li-Fe in the blood of cancer patients, whereas, Cr-Li, Cd-Cr, Mn-Li, Ni-Mn, Cr-Co and Cr-Mn exhibited noteworthy relationships in the blood of controls. Significant differences were observed in the blood metal levels of the patients and controls based on gender, habitat, food habits and smoking. Disparities in the metal levels were also noted for various cancer types (adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell lung cancer) and stages (I, II and III) in patients. Multivariate principal component analysis (PCA) revealed five principal components of the metals for cancer patients and controls but with significantly dissimilar loadings duly supported by the cluster analysis (CA). The study evidenced considerably diverse variations in the blood metal levels of lung cancer patients in comparison with healthy subjects.

Keywords: *Lung cancer; essential/toxic metal; blood; multivariate analysis; AAS, Pakistan*

1. INTRODUCTION

Trace metals poisoning and related health effects manifest a threatening challenge with the advent of rapid industrialization and technological advances. In human body, metals are absorbed via gastrointestinal tract and/or the lungs and circulated through blood stream to different organs for participating in metabolic/biochemical processes [1]. In fact, elevated concentrations of some trace metals or depletion of essential elements may cause various metabolic instabilities [2]. It is known that that some metals are considered not only as carcinogens but also as co-carcinogens [1]; therefore, the issue of metal carcinogenicity is of great interest in occupational medicine and public policy.

Lung cancer is responsible for about over one million deaths globally each year and three million new cases per year are estimated to arise [3, 4]. There are multiple histologic types of lung cancer as classified by conventional light microscopy; non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) are the major types. NSCLC is further classified into three histologic forms; adenocarcinoma, squamous cell carcinoma and large cell carcinoma [5]. Staging of lung cancer is the basis for predicting survival and is a key to clinical trials that compare treatments among the patients [6]. Tobacco smoking is the predominant identified risk factor for lung cancer in both men and women [7]. Other risk factors include asbestos, crystalline silica, radon progeny, radiation, environmental smoke, metals exposure, genetic and non-genetic factors, a diet low in fruit/vegetable intake, and lower educational level [8, 9, 10].

Monitoring the nutritional status of essential metals and exposure to toxic metals in biological samples are of vital importance. However, the choice of the appropriate sample depends on several factors including toxicokinetics, the convenience or invasiveness of the procedure for sampling and the risk of sample contamination [11]. Most of the clinical methods diagnose trace metals deficiencies or environmental/occupational exposure to toxic metals based on the analysis of blood, serum/plasma, hair, nails and/or urine specimens [12]. Blood is one of the most widely used specimens because of ease of sampling. It is the medium of transport for nutrients and toxic metals to and from tissues and, therefore, provides quick and reliable information regarding the metabolism in the human body [2]. Significant differences in the concentration of various trace metals in the blood of cancerous patients compared with the healthy donors have been reported worldwide [13, 14, 15].

Despite the high morality burden for lung cancer globally, only a few studies have examined the effects of trace metals on the risk of lung cancer. There is also a lack of data on the impact of low level environmental exposure to heavy metals on lung cancer patients as well as on cancer progression in Pakistan. Thus there is a dire need to study the inter-relationship of larger number of essential/toxic metals, which could have clinical and diagnostic significance. This study examined the concentrations of selected essential and toxic trace metal concentrations (Fe, Zn, Cu, Sr, Li, Co, Mn, Ni, Cr, Cd and Pb) in the blood of lung cancer patients compared to the healthy subjects with matching age, habitat and food habits. Mutual associations between the metal levels were

assessed by the correlation study, whereas multivariate methods were employed for the apportionment of the metals for the patients and controls. Metal levels were also compared in various types (adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell lung cancer) of lung cancer and respective stages (I, II and III) in the patients. Viable variations in the metal levels with respect to residence, gender, dietary and smoking habits were also assessed. The study intends to investigate whether trace metals had any presumptive benefits in the diagnosis/prognosis of lung cancer.

2. EXPERIMENTAL

2.1. Study Population. A total of 113 lung cancer patients (including 88 males and 25 females) ages between 37 and 75 years were included in the present study, on volunteer basis. Subjects were selected among the patients admitted in Nuclear Oncology & Radiotherapy Institute (NORI), Islamabad, Pakistan. Prior to the sample collection, the protocol of the study was approved by the ethical review committee of the institute. The recruitment criteria for the patients included no prior radiotherapy, chemotherapy or anti-neoplastic treatment and no evidence of lung surgery. Furthermore, they were not taking any mineral supplements for the previous three months. Clinical diagnosis was confirmed by the histopathological and radiological examinations of the patients [6]. All lung cancer patients were newly diagnosed and previously untreated. The subjects were briefed about the objectives of the study and a written signed consent was obtained from each donor before sample collection.

The healthy subjects/control were also selected on volunteer basis from the same localities with matched age groups and similar socioeconomic status/food habits (n = 109). In most of the cases, control was the healthy family members of the patient. The controls were also briefed about the purpose/objectives of the study and then a written consent was obtained. A proforma was filled to record the information such as age, gender, habitat, ailment duration, food habits, smoking habits, type of ailment, medicine, hobbies, occupation and tumour type/grade etc., at the time of sample collection from the subjects of both groups.

2.2. Sample Collection and Preparation. The blood samples were collected from an antecubital vein by using appropriate precautions to prevent exogenous contamination [16]. Approximately 3 mL venous blood was collected in a metal free sterile blood collecting tubes (BD Vacutainer Ref. 366430) [17]. The samples were kept in refrigerator until further processing. Exactly known amount of blood sample was transferred from storage tube to the digestion flask and digested with HNO_3–$HCLO_4$ (10:1 v/v) mixture with subsequent heating to a soft boil until dense white fumes evolved. Samples were then cooled to room temperature [18]. The digested solutions obtained were transferred quantitatively to 50 mL volumetric flasks and diluted with doubly distilled water [19]. To check for possible contamination during the digestion procedure/processing efficacy, a blank solution (without blood sample) was processed with each batch of the 6 samples [20]. All the reagents used were of ultrahigh purity (>99.99%).

2.3. Quantification of the Metals. The quantitative measurement of selected essential and toxic metals (Fe, Zn, Cu, Sr, Li, Co, Mn, Ni, Cr, Cd and Pb) in the digested samples was performed on flame atomic absorption spectrophotometer (Shimadzu AA-670, Japan), with automatic background compensation and under optimum analytical conditions as shown in Table 1. Three sub-samples of each sample were treated and run separately onto the atomic absorption spectrophotometer to pool mean metal concentrations [21]. Parallel routine check on the accuracy of quantified results was ensured through the use of Standard Reference Material, with certified values of the analytes (Bovine Muscle Powder, NIST-SRM 8414), which showed very good recoveries (98–102%). The samples were also analyzed at an independent laboratory for comparison of the results and a maximum of 2% difference was observed in the results of two laboratories. All the reagents used were of ultrahigh purity (certified purity > 99.99%). Doubly distilled water was used throughout the study for the preparation of the samples and standards. Stock solution (1000 mg/L) of each metal was used to prepare the fresh working standards just before the analysis. Generally, the contribution of the blank was < 5% of the measured metal levels in the samples.

2.4. Statistical Analysis. STATISTICA software was used for statistical analyses of the metal data [22]. The quantified results were subjected to univariate and multivariate analysis in order to classify the relationship among the metal levels. Univariate analysis of the data comprised of the basic statistical parameters, including range, mean, median, standard error (SE) and skewness while mutual variations in the metal levels were computed as correlation study. Significant differences in the measured metal levels for the patients and controls were determined using the Student's *t* test. Principal component analysis (PCA) and cluster analysis (CA) were used for the multivariate apportionment of the metals. The PCA is a powerful tool for evolving better mutual relationships among the variables in a given system and for revealing the groups within the data that have common origin. The main use of PCA is to reduce dimensionality of the linearly correlated dataset by using a smaller number of linearly independent, but new variables. These new variables are principles components, each of which is a linear combination of the originally correlated variables [23]. The CA is employed in order to understand the complex nature of the relationships and apportionment among the metals and provides interesting information on metal sources and pathways [24]. This technique is a classification procedure that involves a measurement of the similarity between variables. A purpose of cluster analysis is to discover a system of organizing observation where member of groups/variables share the properties in common. The variables are grouped in the cluster in terms of their nearness or similarity [13].

Table 1. Optimum analytical conditions for the metal analyses along with their detection/quantification limits and certified Vs measured (±SD) concentrations* (µg/g) of the metals in standard reference materials.

Metal	Wavelength (nm)	Slit width (nm)	Limit of Detection (mg/l)	Limit of Quantification (mg/l)	Bovine Muscle Powder, NIST-SRM 8414		
					Certified Level	Measured Level	Recovery (%)
Fe	248.3	0.2	0.006	0.018	71.2	71.60 ± 1.719	100.6
Zn	213.9	0.5	0.002	0.006	142	143.5 ± 3.081	101.1
Cu	324.8	0.5	0.004	0.013	2.4	2.387 ± 0.051	99.5
Sr	460.7	0.5	0.005	0.016	0.052	0.051 ± 0.002	98.1
Li	670.7	0.5	0.003	0.009	-	-	-
Co	240.7	0.2	0.005	0.016	0.007	0.007 ± 0.001	99.2
Mn	279.5	0.4	0.003	0.010	0.37	0.361 ± 0.023	97.6
Ni	232.0	0.15	0.006	0.019	0.05	0.051 ± 0.004	102.0
Cr	357.9	0.5	0.006	0.018	0.071	0.070 ± 0.003	98.6
Cd	228.8	0.3	0.004	0.013	0.013	0.013 ± 0.002	100.6
Pb	217.0	0.3	0.010	0.029	0.38	0.372 ± 0.019	97.9

*Triplicate sub-samples

3. RESULTS AND DISCUSSION

3.1. Characteristics of the Subjects. The demographic data related to the lung cancer patients and healthy donors are presented in Table 2. Subjects in the two groups were closely matched for their age and majority of them (80% patients and 76% controls) were vegetarians in their food habits. The disease was more common in male subjects (78%) than in female donors (22%). Regarding residence locations, 57% of the patient and 51% of the controls were drawn from rural areas. About two-thirds of the patients (67%) used tobacco on a continuous basis in their everyday life, while a majority of the controls (73%) were not addicted of tobacco. Lung cancer patients included in the present study were most commonly suffering from adenocarcinoma (46%), followed by squamous cell carcinoma (30%), small-cell lung cancer (17%) and large cell carcinoma (07%). Thirty percent (30%) patients were diagnosed at stage-I, 44% at stage-II and 26% at stage-III of lung cancer (Table 2).

3.2. Distribution of Metals. Basic statistical parameters related to the distribution of selected essential and toxic metals in the blood of lung cancer patients and healthy subjects are shown in Table 3. Most of the metals exhibited large range in their concentrations as manifested by the minimum and maximum values in both groups. In the case of lung cancer patients, Fe revealed highest mean levels at 353.9 µg/g, followed by Ni (8.967 µg/g), Zn (6.501 µg/g) and Pb (5.275 µg/g). However, relatively lower mean levels were observed for Sr (1.267 µg/g), Cr (1.080 µg/g), Li (0.604 µg/g), Cd (0.425 µg/g) and Mn (0.278 µg/g). Overall, the metal contents in the blood of the patients revealed following decreasing order in their average concentrations: Fe > Ni > Zn > Pb > Co > Cu > Sr > Cr > Li > Cd > Mn. Most of the metals exhibited random distribution as shown by appreciably divergent mean and median values on one hand and large SE values on the other hand. Higher value of SE reflected a large dispersion of metal contents as noted in the cases of Fe, Pb and Ni. Some of the metals (Mn, Cd and Li) exhibited relatively normal distribution pattern, evidenced by comparatively lower SE values. Somewhat lower values of skewness were noted in favour of Cd, Li, Mn and Fe thus manifesting relatively symmetrical distribution pattern of these metals in the patients.

Table 2. Characteristics of the subjects.

Characteristics	Cancer patients (n = 113)	Healthy donors (n = 109)
Age (years)		
Range	37–75	35–72
Mean	58.1	55.9
Gender		
Female	25 (22%)	29 (27%)
Male	88 (78%)	80 (73%)
Diet		
Vegetarian	90 (80%)	83 (76%)
Non-vegetarian	23 (20%)	26 (24%)
Habitat		
Urban	49 (43%)	53 (49%)
Rural	63 (57%)	56 (51%)
Use of tobacco		
Smoking	76 (67%)	80 (73%)
Non-smoking	37 (33%)	29 (27%)
Types of Lung Cancer		
Adenocarcinoma	52 (46%)	
Squamous cell carcinoma	34 (30%)	
Large cell carcinoma	08 (07%)	
Small-cell lung cancer	19 (17%)	
Stages of Lung Cancer		
Stage-I	34 (30%)	
Stage-II	50 (44%)	
Stage-III	29 (26%)	

In the case of healthy donors (Table 3), Fe (279.8 µg/g), Zn (7.018 µg/g), Pb (4.631 µg/g) and Co (4.307 µg/g) emerged as major contributors, followed by, Cr, Ni, and Sr at 2.591, 2.541, and 2.341 µg/g, respectively. On the whole, the decreasing trend for controls revealed following order: Fe > Zn > Pb > Co > Cr > Ni > Sr > Cu > Mn >Li > Cd. Median levels of the metals also followed the same sequence. Most of the metals exhibited large dispersion and asymmetry in their distribution as shown by SE and skewness values. Some of the metals (Cd, Li, Mn and Cu) exhibited comparatively normal distribution, as indicated by rather lower SE values. Small magnitude of skewness for Cu, Fe, Cd and Mn manifested nearly symmetrical distribution of these metals in

the controls. In most of the cases, the difference between mean and median levels of the metals was comparatively lower in the controls than the patients.

Two-tailed Student's *t*-test ($p < 0.05$) of the data revealed that the average levels of Fe, Li, Ni and Cd were significantly higher in the patients compared to the controls; while the mean levels of Zn, Cu and Pb exhibited insignificant differences in the two donor groups. However, Sr, Co, Mn and Cr depicted significantly elevated levels ($p < 0.05$) in the controls. Overall, the distribution behaviour of the selected metals in the blood of healthy subjects remained noticeably diverse compared to the lung cancer patients, which may be attributed to the disproportions of the toxic and essential metals in the patients. Similarly median levels of the metals were also compared by Wilcoxon-rank sum test which revealed statistically significant differences for Li, Co, Mn, Ni, Cr and Cd in the blood of two groups ($p < 0.05$).

Table 3. Statistical distribution of essential and toxic metal concentrations (μg/g) in the blood of lung cancer patients and healthy donors.

	Patients						Healthy Donors						*p*-value
	Min	Max	Mean	Median	SE	Skew	Min	Max	Mean	Median	SE	Skew	
Fe	229.6	456.5	353.9	360.8	7.561	-0.666	225.1	356.2	279.8	280.8	4.248	0.311	<0.05
Zn	3.048	11.62	6.501	6.354	0.228	0.669	2.247	14.53	7.018	6.838	0.296	0.727	*NS
Cu	0.413	3.769	1.647	1.479	0.111	0.923	0.017	2.845	1.296	1.397	0.089	0.235	*NS
Sr	0.046	3.604	1.267	1.132	0.151	1.053	0.024	5.620	2.341	2.399	0.252	0.196	<0.05
Li	0.016	1.325	0.604	0.546	0.067	0.365	0.019	1.073	0.309	0.229	0.050	1.449	<0.05
Co	0.020	15.04	2.737	2.389	0.449	2.934	0.011	14.03	4.307	4.193	0.508	0.905	<0.05
Mn	0.009	0.711	0.278	0.249	0.031	0.432	0.138	2.535	1.005	0.965	0.076	0.569	<0.05
Ni	0.023	24.45	8.967	8.493	0.729	0.772	0.489	7.571	2.541	2.237	0.266	1.202	<0.05
Cr	0.150	3.890	1.080	0.765	0.143	1.877	0.067	9.497	2.591	1.162	0.446	1.239	<0.05
Cd	0.037	0.933	0.425	0.445	0.036	0.148	0.012	0.694	0.289	0.250	0.028	0.510	<0.05
Pb	0.174	19.95	5.275	3.625	0.765	1.402	0.398	12.45	4.631	4.383	0.563	0.694	*NS

*NS – non significant

3.3. Demographic-Based Variations in the Metal Levels

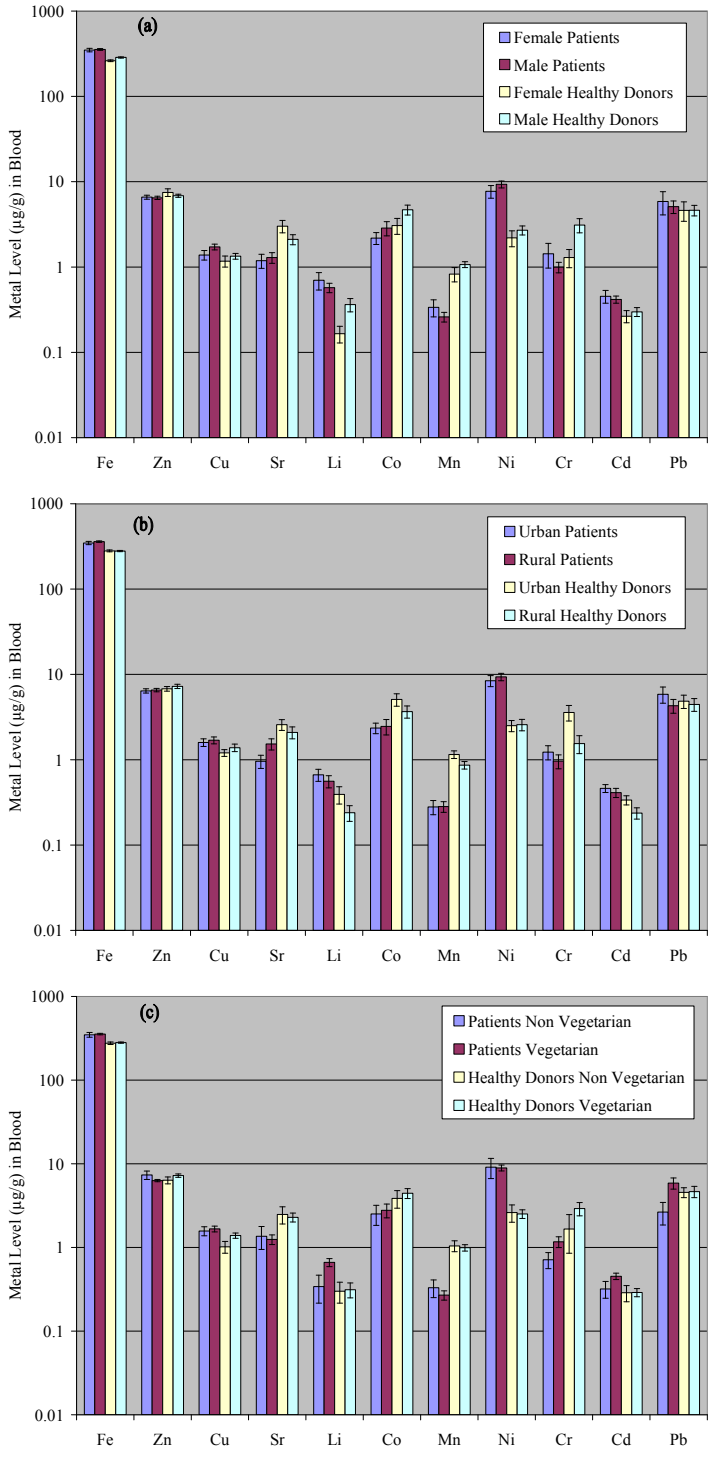
3.3.1. Comparison of the Metal Levels Based on Gender. The gender-based disparities in average metal concentrations in the blood of patients and controls are displayed in Fig. 1a. Average concentrations of Fe, Cu, Li, Ni, Cr, Cd and Pb were found to be relatively higher in the blood of female patients compared to that of the female controls. Nevertheless, Zn, Sr, Mn and Co exhibited appreciably elevated levels in the case of female controls. Moreover, evidently higher levels of Cr, Zn, Sr, Mn and Co were observed in male controls, while Pb, Cd, Ni, Li, Cu and Fe showed significant rise in the case of male patients. Among the metals, Li, Mn, Cr and Cd exhibited similar trend; the metal levels were found to be relatively higher in female patients and male controls, nonetheless; comparatively higher levels were observed for Sr in the male patients and female controls. It was interesting to note that female patients revealed higher Cd levels than the male patients. Cadmium load in gender difference may be due to, in part to the effects of gender specific hormones like estradiol, which raises Cd uptake and accumulation, presumably through induction of metallothionein [25]. Thus these metals might be associated with vital clinical significance related to the disease.

3.3.2. Comparison of the Metal Levels Based on Habitat. Average blood metal levels of the patients and controls inhabiting in urban and rural localities are shown in Fig. 1b. Mean levels of Cu were found to be higher in the blood of rural patients/controls while those of Pb, Cd, Cr and Li were relatively higher in the blood of urban patients/controls. Mean levels of Sr and Ni were noted to be higher for rural patients whereas, Co and Mn contents were higher in the urban controls. For both categories of donors, no significant differences were observed in the levels of Fe and Zn for rural and urban subjects.

3.3.3. Comparison of the Metal Levels Based on Dietary Habit. Comparative average concentration of the metals in the blood of the patients and controls with vegetarian and non-vegetarian food habits are shown in Fig. 1c. Average concentrations of Fe and Ni in the vegetarian and non-vegetarian subjects of both donor groups were not significantly different. However, comparatively higher levels of Sr and Mn were noted in the non-vegetarian subjects and those of Cu, Co and Cr in the vegetarian subjects of both groups. Some metals revealed distinctive behaviour in their trends; mean levels of Zn were found to be somewhat higher in the non-vegetarian patients and vegetarian controls, while average concentrations of Li, Pb and Cd were significantly higher in the vegetarian patients. Thus nutritional habits of the donors may play their part in the prevention or progression of the disease.

3.3.4. Comparison of the Metal Levels Based on Smoking Habit. Mean concentration of the metals in the blood of the donors with smoking and non-smoking habits are shown in Fig. 1d, for comparative evaluation. On the average basis, Fe, Cu, Li, Ni, Cd and Pb levels were considerably higher in the patients with smoking habits, while notably higher concentrations of Sr, Co, Mn, and Cr were observed in the case of smoking controls. Almost similar finding were observed in clinical studies by Kazi et al [14] and Stavrides [7]. It was illustrated that exposure to Cd via cigarette smoke resulted in a dose dependent inhibition of lung clearance and increased absorptions of components of inhaled smoke through the tracheo bronchila airways [1]. Nevertheless, comparative study highlighted the role of smoking in the progression of lung cancerous in terms of Cd levels in the smoking patients. Nonetheless, average concentration of Zn revealed more or less comparable in the patients/controls with smoking habit. In the case of nonsmoking patients and controls, relatively higher contents of Fe, Li, Ni, Cd and Pb were noted in the patients, while significantly elevated mean levels of Sr, Co, Mn and Cr were found in the controls. Average concentrations of Co and Sr were notably higher in the smoking patients and non-smoking controls compared to their respective counterpart donors. Nevertheless,

average concentrations of Fe, Zn and Li in the smoking and non-smoking patients were more or less comparable.



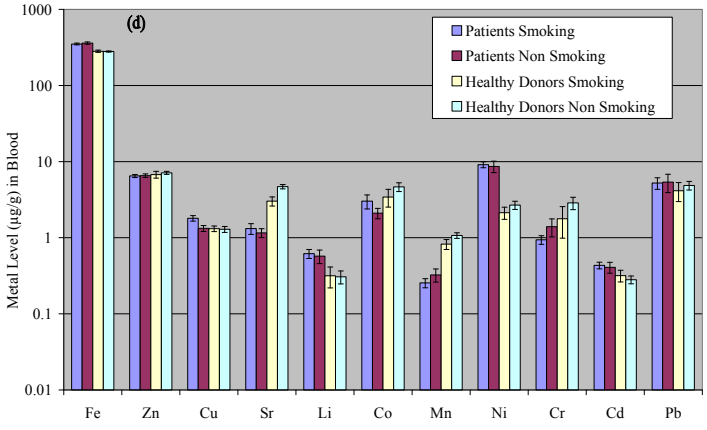


Figure 1. Comparative average concentrations of the essential and toxic metals (±SE) in the blood of lung cancer patients and controls based on (a) gender, (b) habitat, (c) food habits and (d) smoking habits.

3.4. Correlation Study. Spearman correlation coefficients (r) between the metal levels measured in the blood of patients and controls are shown in Table 4 ($p < 0.05$). In the case of patients, strong positive correlations were observed between Pb-Cr (r = 0.714), Cr-Li (r = 0.608), Pb-Co (r = 0.581), Cd-Cr (r = 0.543), Li-Fe (r = 0.523), Cd-Li (r = 0.419), Cr-Fe (r = 0.404) and Cd-Mn (r = 0.398), indicating their probable communal variations/sources in the patients. All other metal pairs exhibited insignificant positive or negative relationships, which manifested their independent variations in the patients. This correlation study revealed that toxic metals demonstrated strong mutual relationships with the essential metals and thus contributed mainly toward the carcinogenic processes. The counterpart data for the controls (Table 4) showed strong correlation coefficients between Cr-Li (r = 0.751), Cd-Cr (r = 0.621), Mn-Li (r = 0.604), Ni-Mn (r = 0.522), Cr-Co (r = 0.518), Cr-Mn (r = 0.511), Li-Cd (r = 0.489), Mn-Cd (r = 0.469), Li-Pb (r = 0.468), Pb-Cd (r = 0.381) and Li-Ni (r = 0.355), thus manifesting mutual variations of these metals. This correlation study revealed that strong correlations among Cr, Cd, Mn, Co, Li, Fe and Ni indicated their common associations in the healthy subjects. A few negative correlations were also observed, but they were not significant. Apparently, positive correlations of toxic trace metals (Pb, Cd, Cr & Li) with essential metals (Ca, Fe, K & Mg) evidenced a build-up of the toxic metals in the cancer patients. Some earlier studies [14, 15, 26] elaborated the role of Pb, Cd and Cr in the development of lung cancer. Thus, the correlation study revealed significantly dissimilar pattern of mutual dependence of the metals in the patients and controls.

Table 4. Correlation coefficient (r)* matrix of essential and toxic metals in the blood of lung cancer patients (below the diagonal) and healthy donors (above the diagonal).

	Fe	Zn	Cu	Sr	Li	Co	Mn	Ni	Cr	Cd	Pb
Fe	1	0.159	0.091	0.011	0.289	0.047	0.071	0.267	0.313	0.229	0.078
Zn	0.221	1	0.258	-0.289	0.035	-0.228	-0.123	0.222	-0.214	-0.032	0.066
Cu	-0.236	0.093	1	0.018	0.123	0.013	0.032	0.173	0.069	0.125	-0.061
Sr	-0.014	-0.199	0.224	1	0.210	0.194	0.295	0.040	0.336	0.254	-0.096
Li	0.523	0.136	0.039	0.294	1	0.106	0.604	0.355	0.751	0.489	0.468
Co	-0.114	0.100	0.257	-0.091	-0.038	1	0.224	0.131	0.518	0.080	-0.121
Mn	0.138	0.143	-0.081	0.197	0.127	-0.078	1	0.522	0.511	0.469	0.041
Ni	0.307	-0.361	-0.183	0.292	0.262	0.070	0.283	1	0.143	0.301	0.116
Cr	0.404	0.076	0.044	-0.104	0.608	0.254	0.205	0.138	1	0.621	0.237
Cd	0.134	0.025	0.023	0.267	0.419	-0.023	0.398	0.088	0.543	1	0.233
Pb	0.164	0.160	-0.002	-0.227	0.293	0.581	0.241	0.079	0.714	0.381	1

*r-values > 0.342 or < –0.342 are significant at $p<0.05$

3.5. Multivariate Apportionment. Another important aspect of the present study was the multivariate apportionment of the metal levels using PCA and CA. The principal component (PC) loadings, extracted using varimax-normalized rotation on the data set for the patients and healthy donors are shown in Table 5. In case of the patients, five PCs with eigen values >1 were extracted, commutatively explaining about 89% of total variance of the data. The corresponding CA based on Ward's method is shown in Fig. 2a, which revealed very strong clusters of Ca–Ni–Na–Sr–Mn, Mg–Fe–K–Cr–Cd–Pb and Zn–Co–Cu–Li. Thus, CA revealed that toxic trace metals share common clusters with the essential metals, evidencing the imbalances of metals in the cancerous patients. The quantitative information regarding multiple relationships of these metals was assessed by PCA in which PC 1 exhibited dominant loadings for Mg, Fe, K, Cr, Cd and Pb. This PC indicated that these metals were originated mostly from the dietary habits, and anthropogenic activities. One important aspect of such grouping is that toxic metals (Cr, Cd and Pb) were grouped together with a main electrolyte (K) of the body. This may lead to malfunctioning in the normal metabolic processes [27]. Additionally, these metals might contribute towards the carcinogenesis. Interestingly, some of these metals are involved in the production of reactive oxygen species (ROS); disrupt the metal ion homeostasis which may lead to oxidative stress [28]. Prolonged and persistent oxidative stress causes changes in cellular redox homeostasis, uncontrolled cell growth and leads to abnormal activation of redox-sensitive signaling molecules, all of which are primary mechanisms involved in metal-mediated carcinogenesis [29]. Similarly, PC 2 exhibited higher loadings for Cu and Ni which were mainly contributed by the external environmental factors. PC 3 for the lung cancer patients revealed dominant loadings of Ca, Na and Cd, while PC 4 exhibited higher loadings for Co and Zn. These two PCs were mainly linked with nutritional habits and anthropogenic contamination. Last PC showed higher loadings for Sr and Mn; all of these metals constituted strong clusters in CA and dietary/nutritional related factors are associated with this cluster of these metals. Overall, PCA results are in good agreement with CA (Fig. 2a). These associations showed altered body metabolism that may later on participate in the onset and progress of the cancer.

The PC loadings, extracted by using varimax-normalized rotation on the data set for controls are also shown in Table 5, which yielded five PCs with eigen values greater than 1, commutatively explaining more than 84% of the total variance of data. The CA of metal data pertaining to the controls is shown in Fig. 2b, which exhibited very strong clusters of Cr–Li–Pb–Cd–Mn. These metals exhibited higher loadings in PC 1, which was mostly contributed by the environmental pollutants particularly, transportation related factors. Higher loadings for Cu and Ni were observed in PC 2 duly supported by CA. Similarly, PC 3 revealed higher loadings of Na, K, Sr and Co, while PC 4 showed elevated loadings for Ca and Mg. CA also showed the mutual cluster of these metals. These metals were mostly contributed by anthropogenic exposure (PC 2), dietary intake and regulated by internal body metabolism of the donors (PC 3, PC 4) [18]. Last PC exhibited significantly higher loadings of Zn and Fe, which also shared a common cluster in CA. Additionally, CA evidenced significant alterations in the metal contents of the patients in comparison with healthy donors. The essential metals K & Na and Ca & Mg revealed a common clusters in controls, while, in the case of the patients, K interfere with Cr while Ni shared a cluster with an essential nutrient, Ca. Likewise, the association of Zn and Fe in healthy donors was noted; however, in the case of patients, Zn shared the cluster with Co instead of Fe. These associations suggested that, in the case of the patients, the metabolism of essential metals was significantly affected by the trace/toxic metals. The multivariate methods, therefore, revealed that the apportionment of metals in the patients was appreciably different compared to the healthy donors, which manifested an imbalance of the metals in the patients. Consequently, the multivariate methods may be used as a diagnostic tool in the clinical studies, and it may provide an alternative technique for the identification as well as onset/prognosis of the disease. To what extent an imbalance in the metal apportionment in the present study can be used as diagnostic tool shall depend on consistency of the observation and its statistical significance, and it seems that further studies on larger sample size may be required to arrive at any deduction.

Table 5. Principal component loadings* of essential and toxic metals in the blood of lung cancer patients and healthy donors.

	Patients					Healthy Donors				
	PC 1	PC 2	PC 3	PC 4	PC 5	PC 1	PC 2	PC 3	PC 4	PC 5
Eigen value	5.382	3.460	2.299	1.205	1.018	4.394	2.914	2.091	1.714	1.634
Total Variance (%)	35.88	23.07	15.32	8.036	6.785	29.29	19.42	13.94	11.43	10.90
Cumulative Variance (%)	35.88	58.95	74.27	82.31	89.09	29.29	48.71	62.66	74.08	84.98
Ca	-	-	0.779	-	-	-	-	-	0.910	-
Mg	0.894	-	-	-	-	-	-	-	0.862	-
Na	-	-	0.740	-	-	-	-	0.731	-	-
K	0.732	-	0.434	-	-	-	-	0.783	-	-
Fe	0.813	0.471	-	-	-	-	-	-	-	0.626
Zn	-	-	-	0.866	-	-	-	-	-	0.919
Cu	-	0.932	-	-	-	-	0.834	-	-	-
Sr	-	-	-	-	0.913	-	0.616	0.619	-	-
Li	0.601	0.481	-	0.426	-	0.962	-	-	-	-
Co	-	-	-	0.890	-	-	-	0.677	-	-
Mn	0.464	-	-	-	0.611	0.760	-	0.431	-	0.408
Ni	-	0.873	-	-	-	-	0.826	-	-	--
Cr	0.952	-	-	-	-	0.949	-	-	-	-
Cd	0.644	-	0.726	-	-	0.725	-	-	-	-
Pb	0.819	-	0.446	-	-	0.801	-	-	0.438	-

*PC loadings <0.400 are omitted

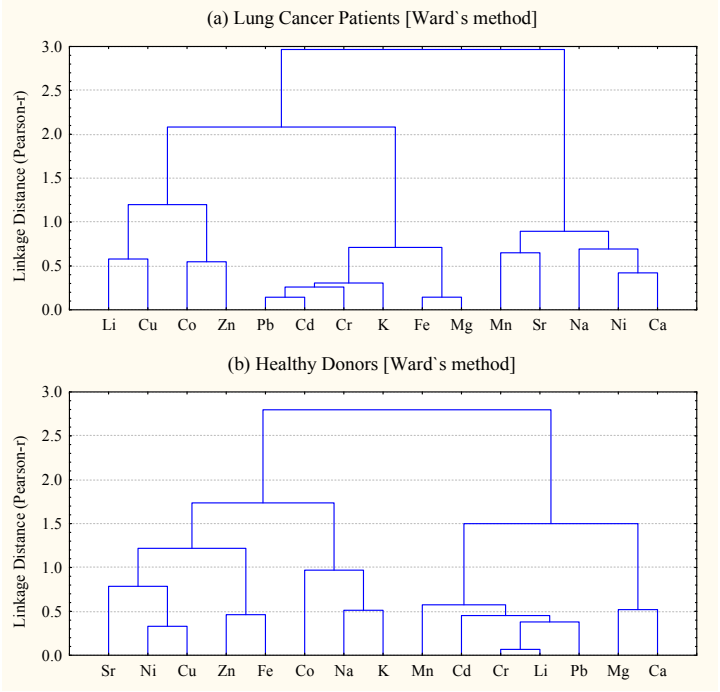


Figure 2. Cluster analysis of the essential and toxic metals in the blood of (a) lung cancer patients and (b) controls.

3.6. Comparison of the Metal Levels Based on Types/Stages of Lung Cancer Patients. Comparative evaluation of mean metal levels in the blood of various types of lung cancer patients (i.e., adinocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell lung cancer) is shown in Fig. 3a. In case of adinocarcinoma patients, Pb and Mn exhibited highest average concentrations, while mean levels of Fe, Sr, Li and Cr were appreciably higher in the squamous cell carcinoma patients. However, only Cd showed highest concentration in the large cell carcinoma patients, while Ni, Co and Cu levels were relatively higher in small cell lung cancer patients. In addition, most of the trace metals (Pb, Mn, Sr, Li, Cr, Cd, Ni & Co) along with redox active metals (Fe & Cu) exhibited significant accumulation in the blood which revealed adverse effect of these metals on the emergence and development of lung cancer [14]. On the other hand, majority of the metals (Fe, Zn, Sr, Li, Co, Mn, Ni, Cr and Pb) exhibited lowest concentrations in the large cell carcinoma patients.

Mean metal levels in the blood of lung cancer patients at different stages are shown in Fig. 3b for comparative evaluation**.** Some of the metals (Fe, Zn and Cu) exhibited approximately comparable levels at all three stages. However, mean levels of Pb, Cd, Cr, Ni and Li were considerably higher at stage-III in the lung cancer patients. Apparently elevated levels of the toxic metals exhibited at stage-III in the lung cancer patients revealing their toxic role in the progression/development of the cancer. Another study [17] also reported higher levels of Cd and Co at stage-III and stage-II in lung cancer tissue. Average concentrations of Co and Mn were relatively higher at stage-II and mean concentration of Sr was found to be relatively higher at stage-I of the lung cancer patients. Likewise, most of the toxic/trace metals (Pb, Cd, Cr, Ni, Li, Co, Mn and Sr) revealed noticeably higher levels at different stages. Similar result for Cd was reported in another study [14]. Among the metals, Pb and Cr exhibited gradual build-up in the blood levels from stage-I to stage-III of lung cancer patients.

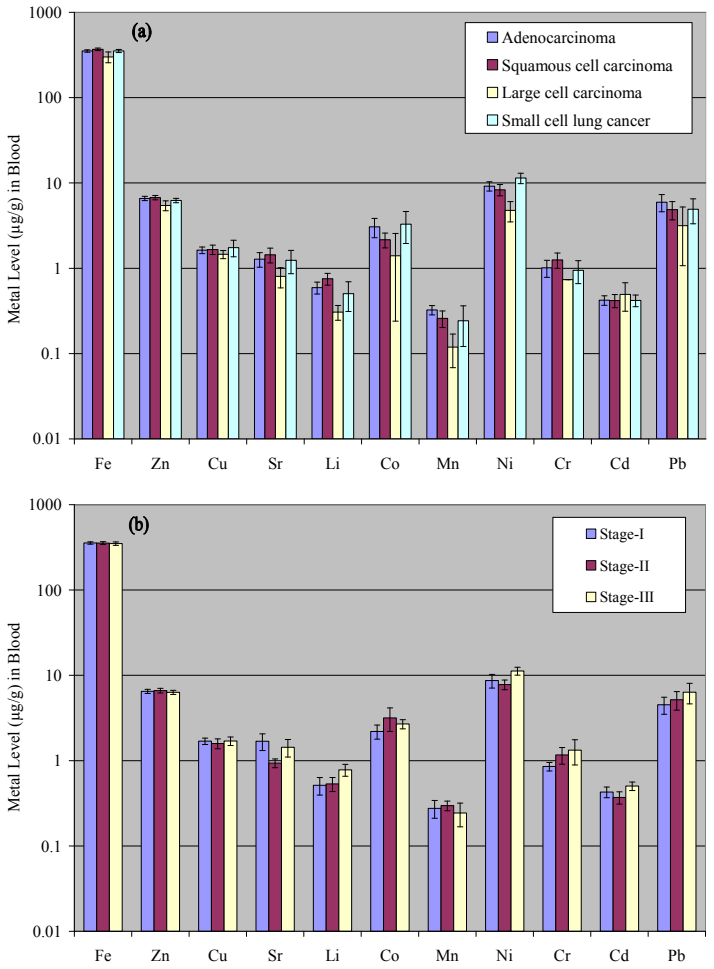


Figure 3. Comparative average concentrations of the essential and toxic metals (±SE) in the blood of lung cancer patients based on (a) various types and (b) stages.

3.7. Metals and Oxidative Stress in Lung Cancer. Metals are mostly bound to proteins, phosphates, polyphenols and other chelating compounds in the biological systems. As constituents of active sites, redox active metals (such as, Cr, Fe, Cu) undergo cycling reactions, participating in the transfer of electrons between metals and substrates/cofactor and play an important role in the maintenance of redox homeostasis. The redox inactive metals obliterate the major antioxidant, especially thiol containing antioxidants and enzymes [30]. Most of the metals exhibit toxicity and carcinogenicity via oxidative stress, which results in uncontrolled metal-mediated formation of deleterious free radicals. These radicals are known to react with all components of DNA, thus damaging its bases and the deoxyribose backbone causing mutations in the crucial genes. Therefore, the process of breakdown of metal-ion homeostasis due to oxidative stress involve in lung cancer [31].

Metals can produce the radicals based on oxygen species (ROS) and up to 1–3% of the pulmonary intake of oxygen by humans is converted into ROS [32]. Under hypoxic conditions, the mitochondrial respiratory chain also produces nitric oxide, which can generate reactive nitrogen species (RNS). ROS are derived from endogenous sources (mitochondria, cytochrome P450 metabolic process, peroxisomes, inflammation etc.) as well as, exogenous sources such as radiation (X-, γ- or cosmic rays, α-particles from radon decay), ions, redox cycling components, environmental agents, metals (redox and non-redox) and solar

light [33]. Moreover, endogenous sources of cellular ROS are neutrophils, esinophils and macrophages [30]. Oxidative damage of DNA is thought to play a critical role in all stages of carcinogenesis [34]; however, the actual effects of oxidative stress may depend on the cellular genetic background, the types of ROS involved, and extent and time of interference of the ROS [35]. Prolonged and persistent oxidative stress causes changes in cellular redox homeostasis, uncontrolled cell growth and leads to abnormal activation of redox-sensitive signalling molecules, all of which are primary mechanisms involved in metal-mediated carcinogenesis [36, 37]. The relationship between oxidative stress and lung cancer has been the subject of intense debate; mainly due to the well documented fact that the cancer cells are under high levels of oxidative stress compared to normal cells [38]. The lungs are directly exposed to oxygen concentrations higher than in most other tissues. ROS in terms of oxidative stress probably enhance the final irreversible stage of carcinogenesis, which is characterized by accumulation of additional genetic damage, leading to the transition of the cell from benign to malignant [39].

Significantly diverse distribution and apportionment of the metals in the blood of lung cancer patients compared to the disease-free healthy group in the present study may be indicative of their possible involvement in the onset/progress of the cancerous disease. It is evident from the data that the demographic characteristics were not significantly different among the patients and healthy donors, indicating the possible relevance of unknown risk factors. A growing body of evidence has indicated that many trace metals play an important role in a number of biological processes by activating or inhibiting enzymes, by competing with other elements and metalloproteins for binding sites, and by affecting the permeability of cell membranes or by other mechanisms. It is, therefore, reasonable to assume that the metals would exert action, directly or indirectly, on the health status. In earlier studies, mean concentrations of Cd, Pb, Ni, Cu and Fe were reported to be significantly higher in the lung cancer patients compared with controls [1, 14, 40, 41].

3.7.1. Cadmium. It is clear that profound variation in Cd, which exist in cigarette smoke and thus inhaled during smoking, can cause lung cancer [7, 14]. Cd-induced oxidative stress appears to play a major role in mediating the negative effects of Cd in the lung in relation to both asthma and pulmonary fibrosis [42, 43]. It is thought that Cd acts via genotoxic mechanisms including induction of single-strand DNA breaks, chromosomal aberrations, chromatid exchanges and produces oxidative damage in plants, mammalian and human cells. The development of lung cancer has also been shown to follow inhalation of Cd in experimental animals [44]. Many occupational cohort studies reported in literature have found statistically significant increased risks of lung cancer associated with relatively high Cd exposure [45, 46]. New research advocates that Cd is one of the critical elements causing emphysema, and even low level exposure attained through second-hand smoke and other means may also enhance the chance of developing lung disease [47]. The results of present study were consistent with the other reports in which Cd concentrations were higher in lung cancer patients compared to the controls, which underscores its toxic contribution towards the progression and/or development of lung cancer [14].

3.7.2. Lead. Lead and lead compounds are involved in lung cancer by several routes of administration in rats and mice [48]. Some cytogenic studies on animals have shown that Pb induces DNA-somatic mutations or strand breaks, although not consistently. Cytogenic studies on exposed workers indicated that Pb and its compounds were genotoxic in humans [49, 50]. Epidemiologic studies have illustrated a possible, but inconsistent, association between occupational exposure to Pb and lung cancer [26, 51]. In the present study, mean Pb content was found to be considerably higher in blood of patients than controls (Table 3), which is consistent with the above mentioned hypothesis. Steenland and Boffetta, [52] reported the most likely indication associating of Pb with lung cancer [14]. Furthermore, Vainio, [53] found evidence of association between long term high exposure to Pb compounds and an increased risk of lung cancer.

3.7.3. Nickel. Nickel is another mutagen, which is also associated with lung cancer [54]. It involves epigenetic alterations, disruption of cellular iron homeostasis, generation of ROS and activation of the hypoxia signalling pathway, inhibition of nucleotide excision repair and an increase in DNA methylation leading to inactivation of gene expression [55]. Epidemiological studies have demonstrated a correlation between the incidence of respiratory cancers, lung and nasal, following worksite exposure to Ni [56]. Significantly elevated Ni concentration in the blood of the patients compared to the healthy subjects was noticed in the presently study, clearly indicating the adverse effect of Ni overload in the patients (Table 3).

3.7.4. Copper. Although Cu is also an essential element, yet its high concentration could induce growth proliferation and cancer, particularly, due to its ability to change between Cu(I) and Cu(II), whereby highly ROSs are generated, which produce hydroxyl radicals that adversely modify proteins, lipids and nucleic acids [57]. Excess Cu has been known to be a potent oxidant causing the generation of ROS in the cells. The elevated oxidative stress in cells can lead to modification of number of cellular targets and cause cell damage and death [37]. The cell damage and the subsequent lack of cellular repair processes due to the constant oxidative damage have been associated with carcinogenesis [58]. Copper might promote tumor growth by stimulating tumor angiogenesis. It has been observed that, from its normal binding site on p53, Zinc is displaced by Cu resulting in protein misfolding and impairment of its function [59]. It is confirmed that Cu levels play an important role in indicating the stage of lung cancer development [47]. Reszka et al., [60] conducted a study on individuals with lung cancer and found markedly elevated Cu level compared to the controls. Moreover, in the present investigation, it was found that Cu contents in cancer patients were appreciably higher than the counterpart healthy donors (Table 3). It may be attributed to the redox active nature of Cu which plays its part in the progression of the disease.

3.7.5. Iron. Iron is essential for the normal physiological functions in humans, since it is an integral part of many proteins and enzymes; but it was proposed that relatively low Fe levels could play a role in the prevention of infection and cancer [47]. The excessive accumulation of Fe in humans may be associated with an increased risk of cancer [61]. It causes tissue damage by acting as a catalyst in the conversion of hydrogen peroxide to free-radical ions, which attack cellular membranes, cause DNA strand breaks,

inactivate enzymes, depolymerize polysaccharides and initiate lipid peroxidation. It promotes inflammation and increase in cancer cell growth [62]. Normally redox active Fe is found in the lung lining fluid but its function is not known. This form of iron can be directly involved in carcinogenesis. However, the level of redox active form in the pulmonary epithelial lining fluid might increase depending on the level of Fe intake, which might make physiological defense more difficult and lead to increased oxidative stress, which could in turn lead to a higher risk of lung cancer [40]. In the present study, it showed markedly higher levels in the blood of patients, which supported the above toxic role of this redox-active metal in the development of lung cancer (Table 3).

3.7.6. Cobalt. Cobalt is genotoxic in-vitro and in-vivo and studies confirmed experimentally that Co can not only interfere with DNA repair processes but can also cause direct induction of DNA damage [30]. Several epidemiological studies reported that occupational exposure of Co was linked to an increased lung cancer risk, [63] but further studies are required for clarification. Moreover, Co has been shown to have a carcinogenic effect in rodents as well [64]. In contrast, the present study showed that mean concentration Co was significantly lower in the lung cancer patients than the healthy donors (Table 3).

3.7.7. Chromium. Induction of lung cancer by Cr was first known as an occupational health hazard in industrial exposure such as tanneries, steel wielding, or Cr plating [65]. Recent evidence advocated that particulate matter containing insoluble Cr is deposited on the epithelial surface of the lung where it accumulates to levels high enough to produce cancer [66]. Rowbotham et al., [67] and Sorahan et al., [68] suggested that Cr was involved in human lung cancer by noting that workers with occupational exposure to Cr have a higher incidence of lung cancer [15]. Inversely, a significantly lower level of Cr was found in lung cancer patients than that in controls in the present investigation (Table 3), which revealed that Cr did not directly or independently associated with the development of lung cancer.

3.7.8. Zinc. Zinc plays an important role in nucleic acid metabolism, cell replication, tissue repair and growth through its function in nucleic acid polymerases [69]. It is also involved with metallothionine synthesis, which is thought to inhibit free radical production [59]. Low concentration of Zn has been associated with growth retardation, cognitive impairment and increasing cancer risk, but in excess it can be neurotoxic [70]. In addition, Cd compete with Zn, it displaces Zn from metallothionein. Donation of Zn by metallothionein and in turn extraction of Cd reduces the toxic burden associated with Cd [71]. Cobanoglu et al., [47] investigated that blood serum Zn level was significantly lower in lung cancer patients compared to the healthy subjects. In addition, in the present study, blood Zn levels were evidently lower in the patients than the controls (Table 3). Decreased gastrointestinal absorption and tissue-specific absorption of Zn may be having contributory effects [47].

3.7.9. Manganese. In humans, Mn is essential for normal physiologic functioning and low level of its exposure in the diet is considered to be nutritionally essential. Chronic effects of Mn reported in humans from inhalation exposure to Mn are respiratory effects such as, an increased incidence of cough, bronchitis, dyspnea and an increased susceptibility to infectious lung disease [47]. Cancer development after Mn exposure were studied in only a few long-term animal studies, all of them indicated that Mn did no present a significant carcinogenic risk [72]. No report has appeared where cancer could be definitely attributed to Mn exposure in the human [73]. In the present study, mean Mn concentration was significantly higher in the blood of healthy donors compared to the patients suffering from lung cancer (Table 3).

Consequently, the current investigation suggested that pathological accumulation of the metals in the blood of lung cancer may be closely related to the process of tumour growth. But due to small number of cases, it was not possible to examine the associations among different histological types and stages of lung cancer possibly induced by the metals. Nonetheless, more studies are needed with wider populations to clarify the effects of the metals on lung cancer risk especially with respect to various types and stages. Possible biological mechanisms should be studied further. Another limitation of the study is that it could not identify the occupational and environmental hazards. Tobacco smoke is a major source of endogenous and exogenous ROS in the lungs. In many advanced countries, tobacco control has been addressed in several comprehensive cancer control plans but efforts must be expanded in the developing countries like Pakistan. Another concern is that the exposure time to environmental carcinogens that are necessary for the development of lung cancer is unknown. It has been reported that the socioeconomic factors also play a role in higher mortality rates in the patients, such as poor nutrition, irregular screening, late diagnosis and unequal access to health care due to poverty. The local hygiene centre facilities are also poor in Pakistan and there are no routine monitoring and screening carried out for those people living in small towns. To prevent health risks, government and environmental protection organizations should take on more stern measures to minimize the emissions of toxic trace metals and monitor their contamination in water, soil and agricultural/food products. Lung cancer education and prevention efforts should also include information on the potential link between lung cancer and the metal exposure.

4. CONCLUSIONS

In conclusion, the present study evidenced marked divergences in the distribution of selected essential and toxic metals in the blood of lung cancer patients in comparison with the controls. The average concentrations of Cr, Mn, Co, Sr and Zn were appreciably higher in the blood of controls; nonetheless, most of the metals (Fe, Cu, Li, Ni, Cd and Pb) revealed relatively higher concentrations in the blood of lung cancer patients than healthy donors. Trace metals also exhibited significant disparities in the blood of patients and healthy donors based on gender, abode, food habits and smoking habits. The correlation study revealed noticeably different accumulation patterns of the metals in the blood of the two groups, which is possibly related to the cancer aetiology or growth stage in the patients. PCA and CA supported the diverse behaviour of the metals in blood of the patients and controls, which evidenced imbalance of the metals in lung cancer patients.

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