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Comparative Assessment of Essential and Toxic Metals in the Blood of Spondyloarthropathy Patients and Healthy Subjects

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Abstract: The present study aims to evaluate selected essential and toxic metal levels in the blood of spondyloarthropathy patients compared to healthy subjects. The blood samples collected from both donor groups were digested in a mixture of nitric/perchloric acid. The metal contents were quantified by atomic absorption spectrometry. The comparative distribution of the metals in patients was significantly different from the healthy subjects. Comparison of the metals data indicated that Cd, Co, Cr, Cu, Fe, and Mn levels were significantly higher in the patients, whereas Ca, Mg, and Zn contents were substantially higher in healthy subjects. The correlation study showed significant positive relationships among Pb-Co, Mn-Cd, Cu-Ca, Pb-Mn, Cr-Co, Mn-Co, and Pb-Cr, while significant negative correlations were noted Zn-Ca, Zn-Fe, and Cd-Ca in the patients. Multivariate PCA and CA indicated mutual associations among the essential and toxic metals in patients, whereas, in healthy subjects, the essential and toxic metals revealed entirely diverse apportionment. Effects of demographic factors such as gender, habitat, food habits, and smoking habits on the distribution of metals in both donor groups were also evaluated. The study revealed significantly diverse distribution, correlation and apportionment of the metals in the patients compared to healthy subjects.

Keywords: metal; blood; spondyloarthropathy; statistical analysis; AAS; Pakistan.

1. Introduction

Spondyloarthropathy (SPA) is a family of chronic inflammatory diseases affecting the spinal structure and joints of the pelvis, arms, legs, shoulders, and hips [1]. It is a common disease of joints/ligaments. It is linked with damage of articular cartilage and chondrocytes; it may involve the formation of osteophyte and classical or non-classical monocytes that can uptake and excrete minerals, metallic ions, and elements [2-4]. Early diagnosis of SPA and remission for a comparatively longer time without medication are major challenges in controlling this disease [5]. It is strongly associated with HLA-B27 (human leukocyte antigen) and considered an autoimmune disease, the etiology of which is not fully understood. Essential metals play a vital role in the production, activation, and functioning of the immune system that shows the importance of the balance of trace metals in the onset and progress of SPA [6]. Nevertheless, toxic metals can disturb normal immune responses by taking part in the immune activation mechanism at cellular levels. The environmental factors, including exposure to toxic metals alongside the genetic factors, play an important role in the onset of autoimmune diseases, including SPA. Environmental and genetic factors are found to be associated in both the clinical presentation and course of SPA [7]; however, in the genetically predisposed populations, it is suggested that environmental factors are significantly contributing to the development of SPA [8-11].

Essential metals play an imperative role in biological processes as many cellular reactions depend on their participation. Some of these metals enhance the efficiency and capability of enzymes by acting as co-factors and prosthetic groups, while deficiency of these essential metals may cause several disorders in humans/animals as they have structural and functional importance in various metabolic processes [12, 13]. Calcium is an important constituent of structure, and its adequate intake is necessary for stronger and healthier bones and joints. It plays a major role in developing and maintaining peak bone mass during childhood and adolescence, reducing the risks of autoimmune and infectious diseases [14, 15]. It has been reported that trace quantities of some metals (such as Mg, Mn, and Zn) are found to provide relief from joint pain [16].

On the other hand, elevated concentrations of some metals (such as Co, Cr, Cu, and Mn) proved toxic [17], while prolonged exposure to Cd and Pb is associated with increased oxidative stress and inflammation [18-19]. It was found that elevated Cd, Co, Cu, Fe, and Pb levels are involved in chromosomal aberrations and translocations, contributing to the genetic factor for SPA [20-22]. Another study [23] showed that Cd could induce/excite free radicals, which cause alterations in antioxidant enzymes. Metals' toxicity is becoming more common, and the situation may worsen as these metals can enter into the food chains [24]; therefore, the accumulation of potentially toxic metals should not be ignored.

Reactive oxygen species (ROS) and other free radicals produce an oxidative burden that is believed to play an important role in the onset and progress of autoimmune diseases, including SPA [25, 26]. One of the most damaging ROS in biological systems is the hydroxyl radical formed via the decomposition of hydrogen peroxide catalyzed by metal ions (the Fenton reaction) [18]. These radicals react with the DNA molecules, forming an 8-OH-Guanine adduct, which is a good biomarker of oxidative stress and a potential biomarker of activation of the autoimmune system causing inflammation [19]. Many trace elements (such as Cu, Zn, and Mn) act as structural components of superoxide dismutase, which fights against free radicals and is considered a primary defense line against damaging superoxide radicals [27, 28]. In some cases, higher levels of Mn give a cytoprotective advantage during inflammation of SPA [28, 29].

In the present study, blood samples were utilized to evaluate essential/toxic metals; it is a valuable specimen for this purpose as it is the transport medium of the metals and nutrients. The analysis of blood provides valuable information about environmental and occupational exposure to toxic metals as well as reliable information and detailed insight about the metabolism of the body [30]. The blood samples were collected from SPA patients and closely matching healthy subjects. Selected essential and toxic metals were quantified by atomic absorption spectrometry, and the metals data were evaluated by descriptive statistics, correlation study, and mutual comparison for SPA patients and healthy subjects. On the other hand, the apportionment of the trace metals in both donor groups was also studied by employing multivariate statistical methods.

2. Materials and Methods

The human ethics review committee initially approved the proposal of this study of the Pakistan Institute of Medical Sciences (PIMS), Islamabad, Pakistan. Spondyloarthropathy (SPA) patients were selected among the patients admitted to the Department of Rheumatology, PIMS, Islamabad, Pakistan. The healthy subjects were selected based upon similar age, gender, habitat, food habits, and smoking habits with respect to SPA patients. In most cases, the healthy subjects were close relatives of patients and lived in the same premises to avoid demographic disparities. The subjects (both patients and healthy subjects) participated in this study on a volunteer basis; the participants were briefed about the aims and objectives of the study, and each of the participants signed a written consent form. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted following the Declaration of Helsinki, and the Ethics Committee of the Pakistan Institute of Medical Sciences (PIMS), Islamabad approved the protocol (QAUC/2012/A331-05).

All SPA patients included in this study were newly diagnosed, and the patient with any other disease or taking any supplements was not considered for this study. Similarly, any person suffering from any illness was not selected as a healthy subject and excluded from the study. A total of 64 SPA patients and 70 healthy subjects were included in this study. The demographic characteristics of SPA patients and related healthy subjects are shown in Table 1. The age ranged from 19-55 years for SPA patients and 18-58 years for healthy subjects. Among the subjects, 70% of the SPA patients and 66% of the controls were male subjects. About three-fourths of the blood samples were collected from SPA patients and healthy subjects living in urban premises, while about one-fourth were from rural backgrounds. The assortment of the subjects based on food habits showed that 31% of the blood samples were collected from SPA patients with vegetarian food habits, while 12% of the blood samples were collected from healthy vegetarian subjects. Most of the patients (72%) and healthy subjects (77%) were not addicted to smoking.

Table 1. Characteristics of spondyloarthropathy (SPA) patients and healthy subjects/controls.

Characteristics	**SPA Patients**	**Controls**
n	64	70
Age (years)		
Range	19-55	18-58
Average	31.49	32.19
Gender		
Male	45 (70%)	46 (66%)
Female	19 (30%)	34 (34%)
Habitat		
Urban	46 (71%)	58 (82%)
Rural	18 (29%)	12 (18%)
Food Habits		
Vegetarian	20 (31%)	08 (11%)
Non-Vegetarian	44 (69%)	62 (89%)
Smoking habit		
Smoking	18 (28%)	16 (23%)
Non-smoking	46 (72%)	54 (77%)

The blood samples were withdrawn from the antecubital vein of the forearm by vein puncture method using a disposable syringe. During sample collection, extensive care was taken to avoid any possible contamination; the skin of the sampling area (antecubital vein of the forearm) of the participants was cleaned with ethanol prior to withdrawing the sample. Each fresh time pair of disposable gloves was used while collecting the blood samples. About 5 mL of blood sample was withdrawn from each participant and stored in blood collection tubes (BD Vacutainer Ref. 366430) at -15°C. These blood collection tubes were pre-evacuated and did not contain any preservative or anticoagulation agent to avoid contamination [30, 31].

The blood samples were solubilized by the wet acid digestion method; the acids used for this purpose were concentrated HNO_3 (65%) and $HClO_4$ (70%). The solubilization method of the blood samples was optimized by using different ratios of these acids, and finally, 1:1 volume ratio was found most suitable. The blood samples were weighed accurately (up to ± 0.1 mg) and transferred to the digestion flasks made up of Pyrex glass. A total of 10 mL of HNO_3 was added to the digestion flask containing the blood sample and left for about five minutes; then, 10 mL of $HClO_4$ was added to the flask. The flasks were covered with pre-cleaned watch glasses and kept for 15 minutes at room temperature. Afterward, the digestion flasks were heated on a hot plate at 85°C until a clear solution was obtained. The samples were then cooled to room temperature and transferred to the volumetric flasks. The final volume of the samples was adjusted to 50 mL by 0.1 M HNO_3. One blank sample was also prepared, along with each batch of five blood samples. The blanks were prepared by adding the same reagents in the same sequence except that they did not contain the sample [30, 31].

The selected essential and toxic trace metals, including Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Pb, and Zn, were measured using a flame atomic absorption spectrophotometer (Shimadzu AA-670, Japan). The instrument has the ability for background compensation that enables it to correct the fluctuations arising from factors other than the sample [32]. Some of the factors such as wavelength, hollow cathode lamp current, slit width, rates of fuel and oxidant on the instrument were optimized independently, and the optimized values of these factors are shown in Table 2. The metal standards used for the instrument's calibration were prepared from a stock solution of 1000 mg/L by successive dilutions [30, 33]. The analytical method was also validated by processing and analyzing the standard reference material (Bovine Muscle Powder NIST-SRM 8414); recoveries of the metals ranged from 97% to 104% of the certified values.

Table 2. Optimum analytical conditions zwere maintained on AAS to analyze selected essential and toxic metals using air-acetylene flame (Shimadzu AA-670, Japan).

Metal	Wavelength (nm)	HCL current (mA)	Slit width (nm)	Fuel-gas flow rate (L/min.)	Limit of detection (µg/L)	Limit of quantification (µg/L)
Ca	422.7	6.0	0.5	2.0	4	11
Cd	228.8	4.0	0.3	1.8	4	10
Co	240.7	6.0	0.2	2.2	4	11
Cr	357.9	5.0	0.5	2.6	6	17
Cu	324.8	3.0	0.5	1.8	4	10
Fe	248.3	8.0	0.2	2.0	6	16
Mg	285.2	4.0	0.5	1.6	1	3
Mn	279.5	5.0	0.4	1.9	3	8
Pb	217.0	7.0	0.3	1.8	8	20
Zn	213.9	4.0	0.5	2.0	2	5

3. Results and Discussion

3.1. Distribution of the metals.

The statistical parameters related to the distribution of selected essential and toxic trace metal levels (µg/g, wet weight) in the blood of spondyloarthropathy (SPA) patients are shown in Table 3. Among the metals, dominant mean levels were found for Fe (392.4 µg/g), Ca (66.07 µg/g), and Mg (28.17 µg/g); followed by relatively lower concentrations of Pb (5.553 µg/g), Zn (5.037 µg/g), Cr (4.173 µg/g), Co (3.695 µg/g) and Cu (1.966 µg/g); however the average concentrations of Mn and Cd were found as 1.569 and 0.608 µg/g, respectively. Fe, Ca, and Mg showed significant dispersion in their distribution among the metals, as depicted by their higher values of SD. The symmetry parameters (skewness and kurtosis) showed relatively symmetric distributions of Cr, Ca, Fe and Mn. Moderate asymmetry was found for Cd, Co, and Cu. In contrast, the highest values of asymmetry were noted for Zn, Mg, and Pb. The data relating to the distribution of selected metals in the blood of healthy subjects (Table 3) exhibited the highest mean concentrations for Fe (346.3 µg/g), followed by Ca (244.4 µg/g) and Mg (36.11 µg/g). Relatively lower concentrations were observed for Zn (6.155 µg/g), Pb (5.380 µg/g), Cr (1.281µg/g), Co (1.143 µg/g), and Cu (1.034 µg/g), while Mn and Cd were estimated at the lowest levels (0.478 and 0.417 µg/g, respectively). Among selected metals, Ca and Fe exhibited predominantly non-Gaussian distribution as revealed by corresponding higher SD. However, Mg, Pb, and Zn showed moderate randomness, followed by comparatively narrow distribution for Cr, Co, Mn, Cu, and Cd. Higher asymmetry in the distribution was noted in the cases of Mn, Pb, Ca, Cr and Mg, as shown by comparatively higher values of skewness/kurtosis for these metals. Nonetheless, Cu and Fe showed almost symmetrical distribution in the blood of healthy subjects. Comparative evaluation of the metals revealed a moderate deficiency of some essential metals and accumulation of toxic metals in the blood of SPA patients.

Table 3. Basic statistical parameters for selected essential and toxic metal concentrations (µg/g) in the blood of SPA patients and healthy subjects.

		Min	Max	Mean	Median	SD	SE	Skew	Kurtosis
SPA Patients	Ca	23.58	102.2	66.07	68.52	14.37	1.920	-0.700	1.006
	Cd	0.066	2.331	0.608	0.481	0.533	0.071	1.887	3.003
	Co	0.717	12.26	3.695	3.191	2.263	0.302	1.330	2.378
	Cr	0.070	11.74	4.173	3.414	2.972	0.397	0.943	0.160
	Cu	0.294	5.148	1.966	2.036	0.869	0.116	0.441	2.407
	Fe	142.7	553.3	392.4	398.1	78.19	10.45	-0.610	1.123
	Mg	9.956	65.34	28.17	28.92	8.187	1.094	0.971	7.459
	Mn	0.241	4.377	1.569	1.511	0.914	0.122	1.150	1.759
	Pb	1.465	17.48	5.553	4.979	2.776	0.371	1.824	5.172
	Zn	0.383	17.19	5.037	4.461	2.138	0.286	3.609	19.50
Healthy Subjects	Ca	15.07	2245	244.4	88.65	355.6	41.91	3.211	14.31
	Cd	0.014	1.884	0.417	0.204	0.460	0.055	1.640	1.948
	Co	0.015	4.829	1.143	0.629	1.159	0.132	1.397	1.150
	Cr	0.077	9.252	1.281	0.762	1.472	0.169	2.966	12.01
	Cu	0.193	2.202	1.034	1.018	0.475	0.053	0.500	-0.411
	Fe	53.34	811.6	346.3	329.8	168.5	19.33	0.448	0.125
	Mg	16.64	138.9	36.11	28.19	20.10	2.247	2.417	7.971
	Mn	0.051	4.068	0.478	0.334	0.567	0.069	4.174	23.82
	Pb	0.498	44.58	5.380	3.273	7.912	0.953	3.743	15.33
	Zn	1.017	27.90	6.155	5.128	4.572	0.535	1.721	5.566

Toxic metals have a significant impact on human health, but usually, it is overlooked; the excessive burden of toxic metals contributes significantly to the disease's global burden. It is necessary to avoid the deficiency of essential elements by a balanced diet or food supplements and decrease the burden of toxic metals. The toxic metals such as Cd and Pb disrupt the normal metabolism of essential metals such as Ca, Mg, Fe, Zn, and other nutrients in the human body. As toxic elements disrupt the functions of essential metals and minerals, changes occur in the immune system at a cellular level that may lead to musculoskeletal symptoms and produce SPA conditions. The comparative evaluation of the average levels (*t*-test at $p < 0.05$) of the metals in the blood of SPA patients and healthy subjects revealed that Cd, Co, Cr, Cu, Fe, and Mn exhibited considerably higher contribution in the blood of SPA patients, which showed increased burden of these metals in the patients. On the other hand, mean levels of Ca, Mg and Zn were found significantly higher in the blood of healthy subjects. The average levels of Pb were found slightly higher in SPA patients compared to the healthy subjects. It has been reported that higher concentrations of Cd, Co, Cr, and Mn can show adverse effects on bone metabolism [34]; their higher concentration in the patients compared to the healthy subjects may have a connection with the onset and progress of SPA. Deficiency of Zn or its lower concentration has been noted in SPA patients. Zinc is involved in cell growth, cell protection, production of neoplastic cells, cartilage repair, and decreasing inflammation; its deficiency may be correlated with SPA; an independent study reported that deficiency of Zn was involved in various malignant transformations [35]. Similarly, Zn also provides defense against reactive oxygen species as these ions are part of superoxide dismutases (SODs) and metalloenzymes, which catalyze the conversion of superoxide radicals to oxygen molecules [36]. These trends indicated that deficiency of Ca, Mg, and Zn had adverse effects in the patients, and it may contribute to the onset and progress of the disease.

3.2. Correlation study.

The correlation coefficients for selected metals in the blood of SPA patients are shown in Table 4. Significantly positive correlations were noted among many metal pairs such as Pb, Co, Mn, Cd, and Cr; Ca was found correlated with Cu. Significantly positive mutual correlations of toxic metals (such as Pb and Cd) with some essential trace metals (such as Cr, Mn, Cu, and Co) indicated interferences of toxic metals in the functioning of essential trace metals. A reported study [37] found a correlation of Pb with essential metals in the blood of osteoarthritis patients; such correlation represented a direct toxic effect of Pb on joint tissues or an indirect increased risk factor of autoimmune diseases of joints via secondary bone modification. A positive relationship of Pb with essential metals in SPA patients suggested a contribution of Pb toxicity in the enhanced frequency of occurrence of the disease. Another study showed a link of Pb toxicity with the increasing frequency of autoimmune diseases; it also contributes to the burden of other diseases [38]. The linear relationship of Ca with Cu can be attributed to the interference of Cu in the metabolism of Ca. The positive associations among toxic and essential metals indicated common contributing sources of these metals. There were some significant negative correlations among the metal pairs, such as Zn-Ca, Zn-Fe, and Cd-Ca. The negative correlation of Ca with Cd indicated that Ca supplementation can be beneficial in SPA patients as a balanced concentration of Ca can decrease the absorption of Cd.

The data on metal-to-metal correlations in the blood of healthy subjects are also shown in Table 4. Some important correlations are given as, Mn-Ca, Mg-Ca, Mn-Cr, Cr-Ca, Mn-Mg, Mg-Cd, Cr-Cd, Mn-Cd, Cu-Cd, Mg-Cu, and Fe-Co. On the other hand, some negative associations were found between Zn-Ca, Zn-Mg, and Zn-Cd. Overall, the correlation pattern of the metals in healthy subjects remained evidently different compared to the patients; these associations may be attributed to the disproportions of the trace metals in SPA patients compared to healthy subjects. The immune system and its reactions against different agents are found to be very susceptible to environmental factors (exposure to toxic metals), which induce and enhance autoimmune diseases like SPA [39]. Concerns about the effects of heavy metals and imbalances of trace metals on immune functions are increasing; for example, correlation of Cr in the above case is important as it has the ability to alter immune response by acting as immune stimulatory or immune-suppressive by affecting T and B lymphocytes, macrophages, production of cytokines and cause hypersensitivity of immune response which is very important in onset and progression of SPA [40].

Table 4. Correlation coefficient* matrix for selected essential and toxic metal levels in the blood of SPA patients and healthy subjects.

		Ca	Cd	Co	Cr	Cu	Fe	Mg	Mn	Pb	Zn
SPA Patients	Ca	1									
	Cd	**-0.358**	1								
	Co	0.004	0.059	1							
	Cr	0.014	-0.102	**0.359**	1						
	Cu	**0.427**	0.233	0.017	-0.045	1					
	Fe	0.209	-0.167	0.135	0.087	0.281	1				
	Mg	0.065	0.019	0.171	0.248	0.079	0.200	1			
	Mn	-0.289	**0.545**	**0.335**	0.133	0.045	-0.097	-0.061	1		
	Pb	-0.053	0.093	**0.568**	**0.334**	0.056	0.130	0.168	**0.411**	1	
	Zn	**-0.390**	0.289	-0.084	0.136	-0.127	**-0.359**	0.277	0.190	-0.055	1
Healthy Subjects	Ca	1									
	Cd	**0.490**	1								
	Co	-0.037	0.188	1							
	Cr	**0.741**	**0.618**	0.119	1						
	Cu	0.183	**0.387**	-0.097	0.144	1					
	Fe	-0.048	0.128	**0.312**	0.111	0.068	1				
	Mg	**0.834**	**0.703**	0.109	**0.657**	**0.328**	0.102	1			
	Mn	**0.876**	**0.489**	-0.042	**0.759**	0.175	0.111	**0.730**	1		
	Pb	0.168	0.074	-0.100	0.000	-0.151	**-0.330**	0.003	-0.083	1	
	Zn	**-0.457**	**-0.427**	-0.148	**-0.361**	**-0.342**	0.018	**-0.430**	**-0.343**	0.212	1

*bold *r*-values are significant at $p < 0.05$.

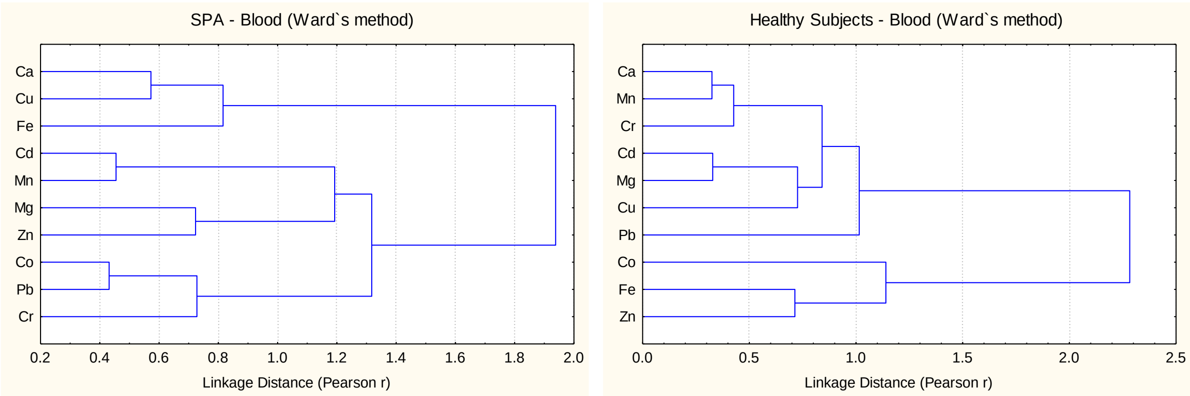
3.3. Multivariate analysis.

Multivariate analysis for the metals in the blood of SPA patients and healthy subjects was performed by principal component analysis (PCA) and cluster analysis (CA). The PCA of metals data was extracted by using varimax-normalized rotation on the data set, as shown in Table 5. Four principal components (PCs) were extracted with eigenvalues greater than one, which cumulatively explained about 71% of the total variance. The corresponding CA based on Ward's method is shown in Figure 1(a), which showed strong clusters of Ca-Cu-Fe, Cd-Mn; Mg-Zn, and Co-Pb-Cr in the patients. The first PC showed higher loadings for Co, Cr, and Pb supported by a strong cluster of these metals in CA. These metals were traced in environmental conditions especially due to anthropogenic activities. These metals are involved in increased oxidative stress and resulted in a higher frequency of autoimmune inflammatory diseases [41]. The second PC showed higher loadings for Ca, Cu, and Fe, and the results are in good agreement with the CA. These metals are involved in various important functions at cellular levels; Cu and Fe have unpaired electrons in their ionic forms, take part in the redox reactions, and perform decisive roles in maintaining human health as part of many cellular redox reactions [42]. Cu, along with Zn, is a physiological component of the antioxidant defense system; they have antioxidant functions through different mechanisms. As a part of intracellular and extracellular antioxidant enzyme superoxide dismutase, Cu-Zn also takes part in the initiation of metallothionein that hunts oxidants and binds redox-active metals [43]. Some of the metals (such as Ca, Cu, Fe, and Zn) are mostly contributed by dietary habits and regulated by internal body metabolism. The third PC indicated higher loadings for Cd and Mn in the blood of the

SPA patients; these metals were controlled by environmental pollutants and contaminated food/drinking water. The fourth PC showed higher loadings for Mg and Zn; these metals are regulated by internal body metabolism and dietary factors. Overall, PCA findings were in very good agreement with the CA results.

Table 5. Principal component analysis of selected essential and toxic metals in the blood of SPA patients and healthy subjects.

	SPA Patients				**Healthy Subjects**			
	PC 1	**PC 2**	**PC 3**	**PC 4**	**PC 1**	**PC 2**	**PC 3**	**PC 4**
Eigen value	2.361	2.094	1.410	1.229	3.901	1.279	1.109	1.049
Total Variance (%)	23.61	20.94	14.10	12.29	39.01	12.79	11.09	10.49
Cumulative Variance (%)	23.61	44.55	58.65	70.93	39.01	51.80	62.89	73.38
Ca	-0.068	0.728	-0.316	-0.046	0.872	-0.101	-0.207	0.016
Cd	-0.025	-0.083	0.904	0.074	0.586	-0.340	0.097	-0.520
Co	0.829	0.074	0.076	0.011	-0.037	0.930	-0.035	-0.018
Cr	0.602	-0.051	-0.194	0.419	0.776	0.004	0.057	-0.186
Cu	-0.110	0.799	0.411	0.086	0.488	0.315	-0.033	-0.354
Fe	0.224	0.628	-0.164	0.034	0.043	-0.095	0.943	0.079
Mg	0.161	0.197	-0.057	0.843	0.668	-0.312	0.013	-0.402
Mn	0.459	-0.175	0.717	-0.102	0.822	0.126	0.091	0.037
Pb	0.821	0.068	0.175	0.006	-0.016	-0.063	0.109	0.881
Zn	-0.147	-0.486	0.323	0.661	0.634	-0.228	-0.504	-0.038



(**a**) (**b**)

Figure 1. Cluster analysis of selected essential and toxic metals in the blood of (**a**) SPA patients; (**b**) healthy subjects.

The PCA of selected metals in the blood of healthy subjects, extracted by using varimax normalized rotation on the data-set, is also shown in Table 5. It showed four significant PCs with eigenvalues greater than one and explaining the cumulative variance of 73.38%. The corresponding CA based on Ward's method in the form of a dendrogram is shown in Figure 1(b). The CA of the metals data in the blood of healthy subjects revealed very strong clusters of Ca-Mn-Cr and Cd-Mg-Cu; other notable clusters comprised of Fe-Zn. In the PCA, PC 1 showed higher loadings for Ca, Cd, Cr, Mg, Mn, and Zn, well supported by CA findings as the first two clusters in CA consisted of these elements. The metals in this group can be traced to environmental pollution and dietary factors. PC 2, 3, and 4 showed higher loadings for Co, Fe, and Pb, respectively; it suggested independent variations of these metals in the blood of healthy subjects. These results were found well supported by CA. These findings evidenced that the origin/sources of essential and toxic metals in the patients and healthy subjects were significantly dissimilar, probably due to the difference in metabolism due to disease conditions.

These associations also indicated that the metabolism of essential metals was significantly affected by toxic metals in the case of SPA patients [44].

3.4. Gender-based comparison.

There are increasing shreds of evidence that the health effects of toxic metals may differ in occurrence and manifestation based on gender [32]. It has been noted that autoimmune diseases, including SPA, are more common in women than in men; the factors related to such gender bias included higher immune system reactivity in females than males [45]. Variations in average levels of the metals in SPA patients' blood compared with healthy donors based on their gender are shown in Figure 2. It was noted that overall, in the blood of female SPA patients, most of the metals were found at higher levels than the male patients; it may have a connection with the fact that females are more commonly suffering from autoimmune inflammatory diseases than males [45]. Similarly, the average levels of Co, Cr, Cu, Mn, and Pb were noted significantly higher in the blood of male patients compared with the healthy male subjects. Nevertheless, measured levels of Zn were found almost equivalent in both donor groups. Likewise, female patients exhibited relatively higher average concentrations of Cd, Co, Cr, Cu, Fe, and Mn than the healthy female subjects, while elevated levels of Ca, Pb, and Zn were noted in the blood of healthy females subjects compared with the female SPA patients. Nonetheless, the average level of Mg was noted comparable in both donor groups. Although Fe, Cu, and Mn are considered essential metals, their excess or deficiency leads to many malfunctions in the body [44].

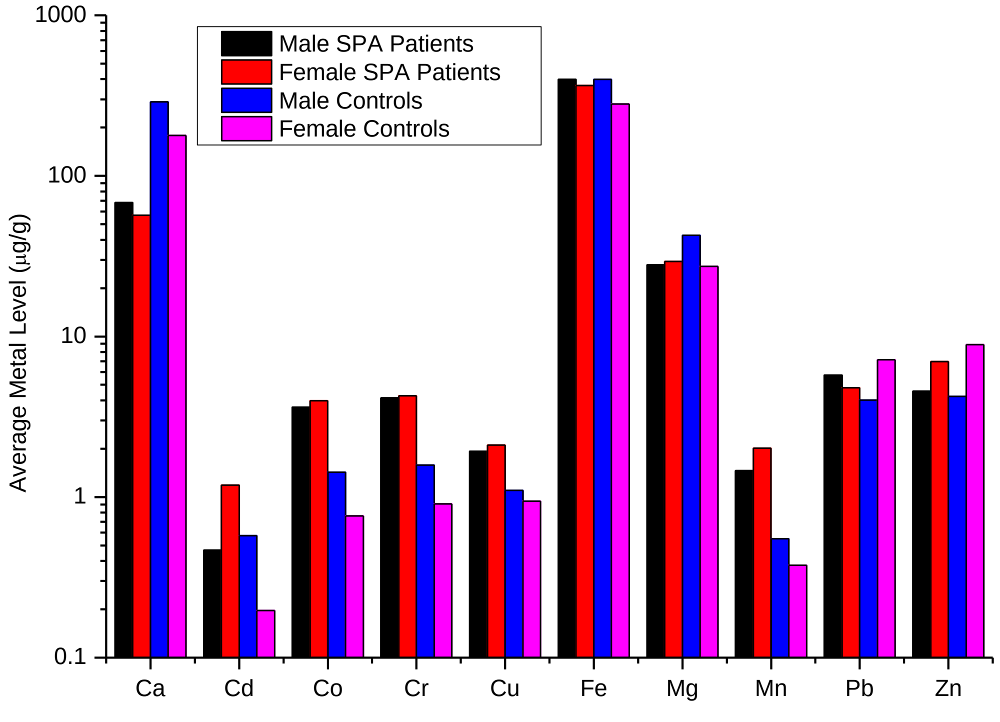


Figure 2. Comparison of average metal levels in the blood of SPA patients and controls based on gender.

3.5. Habitat-based comparison.

Due to anthropogenic activities such as burning fossil fuels, crushing of stones, metals-based pesticides, poultry, animal industry, and metallurgy, there are increased chances of exposure to toxic metals [46]. Average levels of the metals in the blood of SPA patients from rural and urban residential premises compared with similar healthy subjects are shown in

Figure 3. Average levels of most metals were found relatively higher in the blood of the SPA patients from urban areas. The mean levels of Cd, Co, Cr, Cu, Fe, and Mn were found relatively higher in the blood of urban SPA patients in comparison with the healthy urban subjects, while average levels of Pb were found comparable in both groups. These results pointed out the increased burden of the toxic/trace metals in the blood of urban SPA patients; it indicated the role of imbalances of trace metals in the onset of the disease. Similar trends were observed in comparing average metals in the rural SPA patients and healthy counterpart subjects; however, in this case, the sample size is comparatively smaller; hence, further study with a larger sample size is suggested. These trends reinforced the idea of imbalances of metals due to the disease. These results indicated the increased burden of trace metals in urban populations, which may be due to anthropogenic activities, including pollution from vehicular exhaust and industries in the premises of urban areas. Due to human activities, trace metals' biochemical and geological cycles have been altered significantly [47].

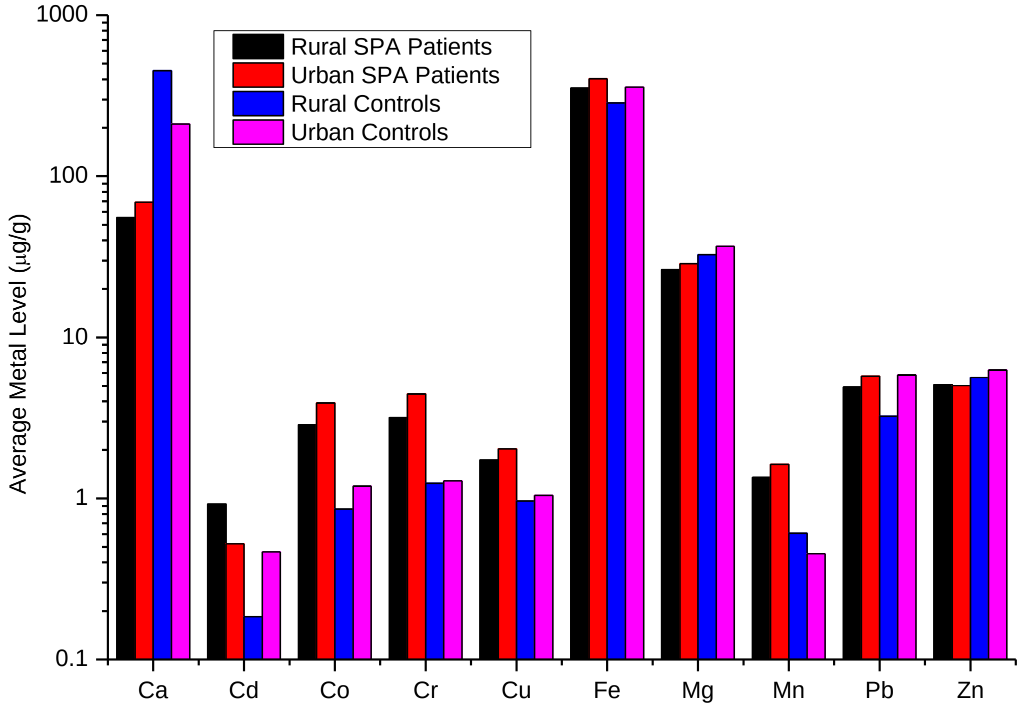


Figure 3. Comparison of average metal levels in the blood of SPA patients and controls based on habitat.

3.6. Diet-based comparison.

Essential and toxic metals have multiple uses, such as industrial, agricultural, and domestic, which is why their wide distribution; the toxicity of a metal depends primarily on the dose, route of exposure, gender, age, and individual nutritional status [48]. One of the major routes of exposure to metals in the body is through ingestion. Hence, food habits play a vital role in the distribution of trace metals in the body. Variations in selected metal levels in the blood of SPA patients with vegetarian/non-vegetarian food habits compared with healthy counterpart subjects are shown in Figure 4. Ca and Mg were found significantly higher in the blood of non-vegetarian healthy subjects than all other groups based on dietary habits; Ca and Mg are very important in bone and joint health, supplementation containing these minerals can be beneficial in SPA patients. Similarly, compared with the healthy vegetarian subjects, the average levels of Ca, Cd, Co, Cr, Cu, Mn, and Pb were considerably higher in the blood of vegetarian SPA patients; however, Fe and Mg levels were found almost comparable in both groups. Comparison of the metal levels in non-vegetarian SPA patients and healthy counterpart subjects showed higher concentrations of Cd, Co, Cr, Cu, Fe, Mn, and Pb in the blood of non-vegetarian SPA patients; while average concentrations of Ca, Mg, and Zn were found noticeably higher in non-vegetarian healthy subjects. Generally, the study depicted significantly different mean values of the metals in the blood of vegetarian and non-vegetarian SPA patients than the matching healthy subjects.

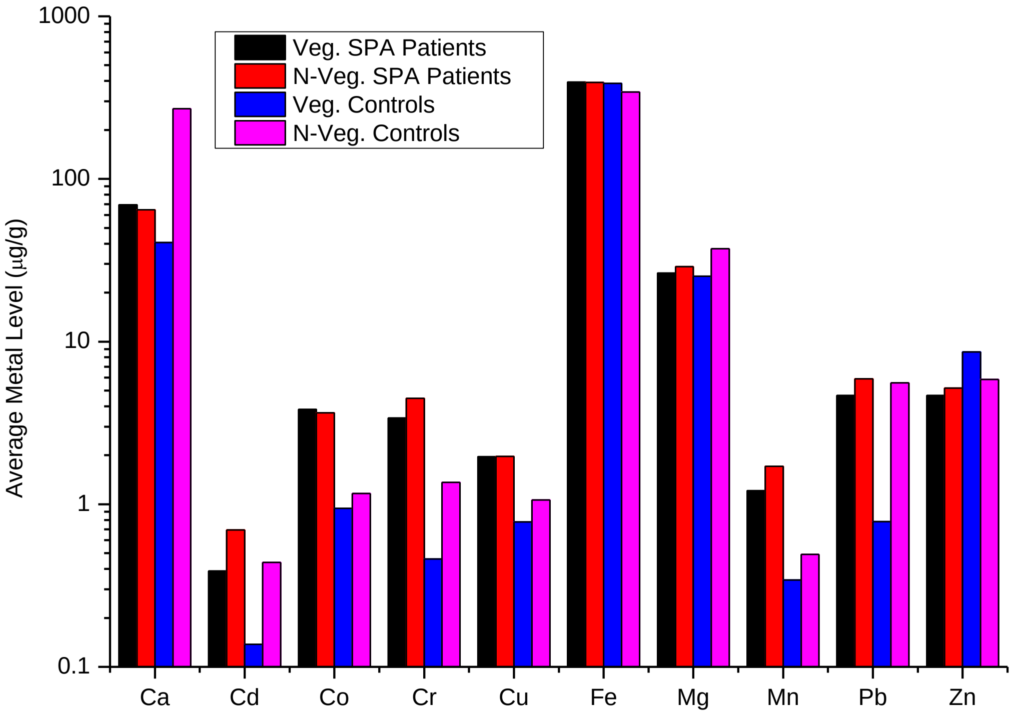


Figure 4. Comparison of average metal levels in the blood of SPA patients and controls based on diet.

3.7. Smoking-based comparison.

Average levels of the metals in the blood of SPA patients with smoking/non-smoking habits compared with similar healthy subjects are shown in Figure 5. Many epidemiological studies have shown an association of smoking habits with an increased rate of various types of arthritis such as osteoarthritis and rheumatoid arthritis. It is considered that excessive use of tobacco may excite the immune system that may cause autoimmune diseases [49, 50]. The comparative study also revealed that average levels of Cd, Co, Cr, Cu, Mn, and Pb were noticeably elevated in the blood of SPA patients with smoking habits in comparison to healthy subjects with smoking habits; while the healthy subjects with smoking habits showed higher average levels of Ca, Fe and Mg in comparison with the smoking SPA patients. Likewise, the patients with non-smoking habits showed higher average concentrations of Cd, Co, Cr, Cu, Fe, and Mn in comparison with the similar non-smoking healthy subjects, which in turn showed comparatively higher average levels of Ca, Mg, and Zn in their blood. However, the average concentration of Pb was found almost comparable in both donor groups. It has been reported that toxic metals exposure by cigarette smoke may increase the risks of autoimmune and inflammatory diseases [51].

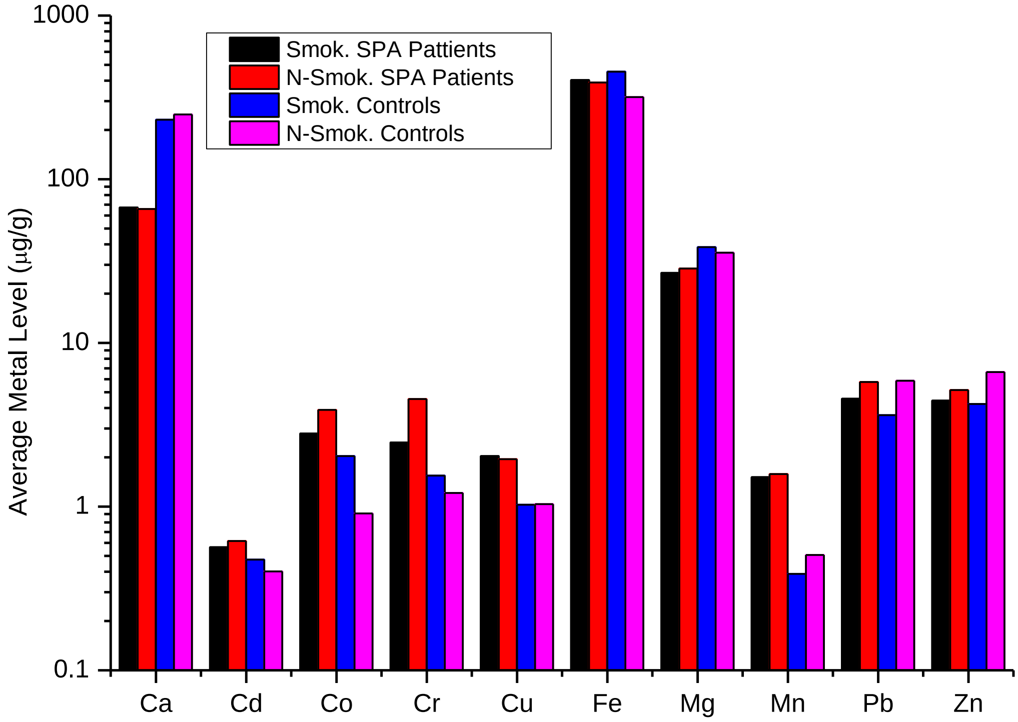


Figure 5. Comparison of average metal levels in the blood of SPA patients and controls based on smoking habits.

3.8. Variations of the metal levels with age.

Variations in the measured metal levels in the patients' blood and healthy subjects were also evaluated on a comparative basis with respect to their ages as metals' uptake and retention capabilities vary considerably among different age groups [30, 32]. Among the selected metals, Cd and Zn levels in the blood showed significantly dissimilar variations with the age of SPA patients and healthy subjects; however, none of the other metals exhibited any viable relationship with the age of the patients and/or healthy subjects. The variations in the measured levels of Zn and Cd in SPA patients' blood compared to the healthy subjects are shown in Figures 6a and 6b, respectively. It was observed that Zn showed an important increasing trend in the blood of both SPA patients and healthy subjects, but the extent of increase was more prominently higher in healthy subjects compared to the SPA patients, which showed only a slight increase with age. It indicated that the concentration of Zn increases more dominantly in the blood of healthy subjects compared to the SPA patients. The deficiency of Zn is associated with many physiological disorders, including arthritic diseases; Zn helps produce neoplastic cells and is considered a protector of the cells [35, 52]. However, the fluctuations in the Cd levels with the age of both donor groups were significantly diverse; the SPA patients revealed a significant increasing trend or accumulation of the Cd levels with increasing age, whereas the healthy subjects exhibited a decline of the Cd levels with increasing age and the decrease in the Cd contents were more significant (Figure 6b). Cadmium can disrupt many biological reactions at much lower doses compared to other toxic metals. It is well known that Cd interferes with the functions of other essential metals, and it is also involved in the production of free radicals, therefore producing an oxidation burden during autoimmune diseases [23, 53]. The increasing trend of Cd levels with the age of the patients can therefore be associated with the onset and progression of the disease.

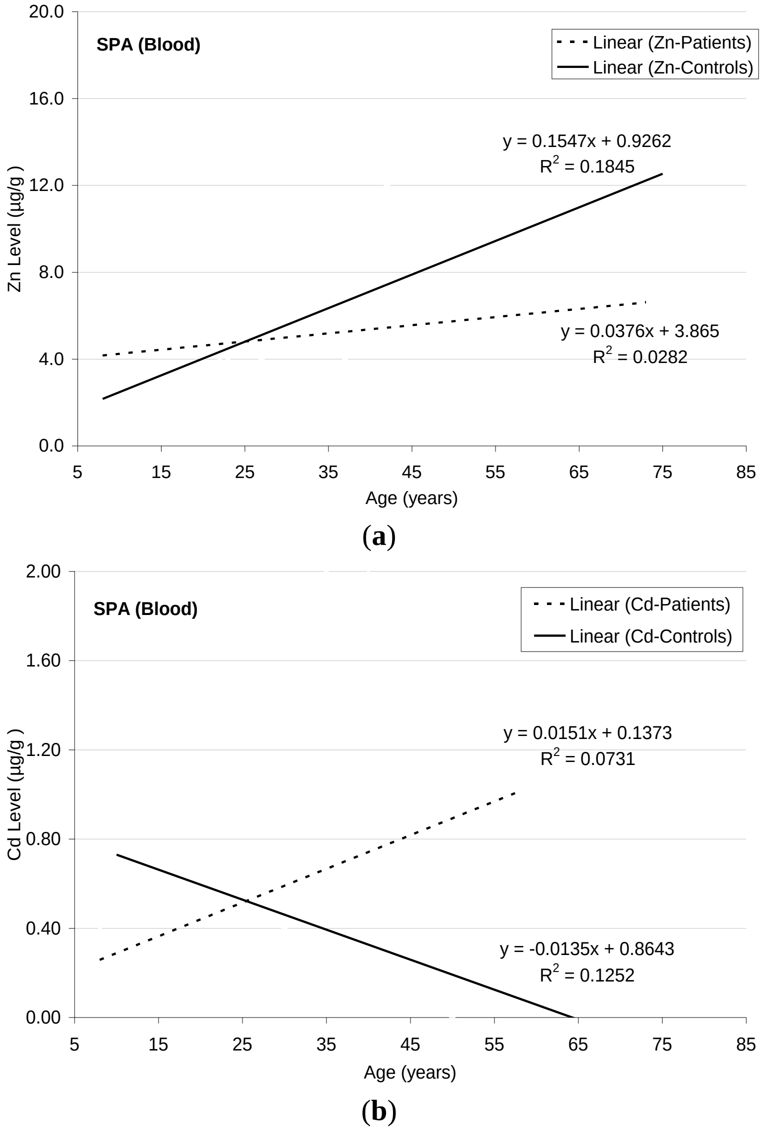


Figure 6. Comparative variations of (**a**) Zn; (**b**) Cd levels with age in the blood of SPA patients and controls.

4. Conclusions

This study showed an increased burden of toxic metals in SPA patients. Considerably higher levels of other trace metals compared to the healthy subjects. In contrast, healthy subjects depicted higher contributions of essential metals (such as Ca, Mg and Zn). Distribution of the metals in SPA patients and the healthy subjects based on gender, habitat, food habits, and smoking habits showed diverse distribution in various groups. Mutual correlations of the metals showed that toxic metals were more associated with essential metals in SPA patients than the healthy subjects. Variations of the metal levels with the age of subjects indicated an increasing level of Zn in the blood of the healthy subjects; whereas, Cd exhibited significantly higher accumulation in the blood of SPA patients; thus, increased levels of toxic metals and decreased levels of essential metals are associated with SPA. Therefore, it is concluded that metal levels may play an important role in the onset and progress of SPA. Intake of certain antioxidants and micronutrients, especially Zn, may play a protective role against SPA.

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Conflicts of Interest

The authors declare no conflict of interest.

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