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Study of imbalances of essential/toxic metals in the blood of osteoarthritis patients in comparison with healthy subjects

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ABSTRACT

Osteoarthritis (OA) is a very common type of joint disease which causes mechanical abnormalities and degradation of joints, articular cartilage and the sub-chondral bone. The present study is based on the measurement of selected essential and toxic metals (Ca, Mg, Fe, Zn, Cu, Co, Mn, Cr, Cd and Pb) in the blood of OA patients and matching healthy subjects/controls. The blood samples were collected form newly diagnosed OA patients and healthy subjects. The samples were solubilized by wet acid digestion method using concentrated HNO_3 and $HClO_4$, followed by the quantification of the metals by atomic absorption spectrometry. Average levels of Cd, Co, Cr, Mn and Pb were found to be significantly higher in the blood of OA patients compared to the healthy subjects, which exhibited comparatively higher concentrations of Cu, Fe, Mg and Zn. The spearman correlation coefficients were calculated for the metals; these were significantly diverse in OA patients and healthy subjects. Multivariate analysis indicated markedly divergent apportionment of the metals in the blood of OA patients compared to the healthy subjects. Plausible variations in the metal levels with respect to gender, habitat, food habits and smoking habits were also evaluated in both donor groups. Overall, the study revealed that the distribution, mutual correlations and multivariate apportionment of the essential and toxic metals in the blood of OA patients was significantly different compared to the healthy subjects.

Keywords: *Osteoarthritis; Blood; Trace Metal; Statistical Analysis; Correlation; AAS; Pakistan*

1. INTRODUCTION

Osteoarthritis (OA) is one of the most common types of the joint diseases [1]. It is an autoimmune disease, which includes mechanical abnormalities involving degradation of joints, articular cartilage and the sub-chondral bone next to it [2-4]. Clinical symptoms of osteoarthritis may include joint pain, tenderness/stiffness, inflammation, creaking and locking of the joints [3]. This disease commonly affects hands, feet, spine, and the weight bearing larger joints, such as hips and knees, although any joint in the body can be affected. As the disease progresses, the affected joints appear swollen, stiff and painful. The diagnosis of OA can often be made with reasonable certainty by clinical examination and through X-rays. Treatment of osteoarthritis patients is often aimed to relieve the pain and restore functioning [5]. Acetaminophen and non-steroidal anti-inflammatory drugs are widely prescribed for the treatment of osteoarthritis [4]. There is a tremendous need to develop potent drugs that either arrest the progression of osteoarthritis or prevent the development of the disease [6]. The exact causes and aetiology of OA disease is complex and not understood properly; among causative agents environmental and genetic factors are considered very important [7].

It has been observed that OA is linked with increased levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Many trace metals such as Cd, Hg, Pb, Fe, As etc. and other agents such as smoking, alcohol, vehicle exhaust, industrial solvents, ozone and pesticides interact in the body to produce ROS and RNS [8, 9]. Nitric oxide radical can start a large number of catabolic reactions which produce inflammatory cytokines [10]. Cellular metabolism also produces ROS as a by-product; over production of ROS may cause number of diseases including osteoarthritis [11]. There are various types of ROS however, the superoxide anion radical and hydroxyl radical have important characteristics in inflammations specially inflammation diseases of joints [12]. Antioxidants are required to scavenge these free radicals; many essential metals directly or with the help of enzymes act as antioxidants thus helping in getting relief from synovium inflammation and to treat the structural changes taking place in the joints [13]. Many antioxidants such as flavonoids, vitamin-E, carotenoids, vitamin-C and trace metals cannot be prepared inside the body, thus these are to be taken from the diet or supplements [14].

Some trace metals are of prime importance in biological systems in providing structural support, helping in conduction of nerve impulse, working of muscles, production and functioning of hormones and enzymes [15]. Study of trace metals is of special interest due to their toxic and essential nature [16]. Many metals are considered as micronutrients and their deficiency may cause clinical manifestations but the same metals are toxic if present above than threshold values [17, 18]. Balance among the concentrations of trace metals is recognized to be essential to normal human homeostasis [19-21]. Metal ions also play essential roles in about one third of the enzymes [22, 23]. A study indicated that natural multi-mineral supplements exhibited anti-inflammatory and anti-oxidant properties [24]. Another study indicated the increased daily consumption of magnesium caused reduction in inflammation around the affected joint [25]. Similarly, low level of zinc may cause osteoporosis, poor outcome of pregnancy and congenital diseases [27]. Therefore, it is anticipated that the metal imbalances may have significant role in onset of OA disease [26].

Understanding the effects of essential and toxic metals on human health is as complex as it is fascinating; the elevated metal concentrations may prove toxic while the depletion of some trace metal levels may cause various metabolic instabilities [28-32]. Many diseases of previously unknown aetiology now are ascribed to the imbalances of trace metals [30, 31]. Human health can be maintained only when the intake of food, water and air provides the optimal daily doses of all essential elements [33]. The excess or deficiencies of trace metals have been associated with many diseases but the exact mechanisms of metal induced ailments have been scarcely explained [34]. Trace metal analysis is utilized more often nowadays in the biomedical field for diagnostic purposes and for evaluation of environmental/occupational exposure [35]. Thus, there is a vital need for the measurement essential and toxic trace metals in the biological specimens to examine the relationship between the diseases and imbalances among the metals. Keeping these aspects in mind, concentrations of selected essential and toxic metals (Ca, Mg, Fe, Zn, Cu, Co, Mn, Cr, Cd and Pb) in the blood of OA patients and counterpart healthy subjects were evaluated. The present study is mainly focused to understand the comparative variations/imbalances in the concentrations of trace metals in the patients and controls.

2. EXPERIMENTAL

2.1. Selection of the subjects. Newly diagnosed OA patients (n = 71) and matching healthy subjects (n = 82) were included in this study on volunteer basis. The patients were selected among the subjects admitted in Department of Rheumatology, Pakistan Institute of Medical Sciences (PIMS), Islamabad, Pakistan. An approval of this study was sought from ethical review committee of the institute before sample collection. The healthy subjects or controls were selected from close relatives of the patients with matching age, gender, food habits and habitat. The subjects (both patients and controls) were initially briefed about the purpose and objectives of the study. The information about sample collection, discomfort and any risks involved were briefed to the participants before collecting blood samples. All the subjects participated in this study on a volunteer basis and a written consent was obtained from each participant before sampling. Important information such as name, age, gender, habitat, food habits, smoking habits, occupation, socioeconomic status, type of ailment, ailment duration, stage of the disease, medication and mineral supplements, etc. related to the subjects were noted on a proforma at the time of sample collection.

2.2. Sample collection. Measurement of the metal levels in the blood provides real insight into the transport of trace metals in the body [36]. The blood samples were collected from antecubital vein of forearm using disposable syringe as per standard methodology. About 5.0 mL of the blood sample was drawn by the syringe and transferred to the sample collection tubes (BD Vacutainer Ref. 366430). These blood samples were stored at -15°C till further processing. Every care was taken to avoid the possibility of contamination during the sample collection. The sample collecting tubes (BD Vacutainer) did not contain any preservative or anti-coagulant agents; thus this method was helpful to avoid any chemical contamination from the sample storing equipment and found suitable for accomplishing the accurate results of trace metals concentrations [15].

2.3. Sample preparation. Wet acid digestion was applied to the blood samples for their complete solubilisation; concentrated HNO_3 (65%) and $HClO_4$ (70%) were used in 1:1 (v/v) for this purpose. The method was optimized previously by using different proportions of the acids. It involved transfer of the blood sample from storage tube to the digestion flask (Pyrex glass) and weighed accurately (up to ± 0.1 mg). To the flask containing blood sample, 10 mL of concentrated HNO_3 (65%) was added and the flask contents were left undisturbed for 5-10 minutes; afterward 10 mL of concentrated $HClO_4$ (70%) was added and left the sample undisturbed for another 5-10 minutes. The flask contents were then heated gradually on hot plate (up to 80°C) for complete mineralization until a clear solution was obtained. The sample contents were then cooled to room temperature, and transferred to the volumetric flasks and diluted up to the mark (50 mL) with doubly distilled water. Similarly, a blank was also processed in the same manner along with each batch of 5-8 samples. The blank contained all the reagents in the same sequence and followed by the same steps except that the blood sample was not present in it. The blank contribution of each metal was subtracted from the sample results to assure the quality of the metals data [15].

Table 1. Optimum analytical conditions maintained on AAS for the analyses of selected metals using air-acetylene flame.

Metal	Wavelength (nm)	HC lamp current (mA)	Slit width (nm)	Fuel-gas flow rate (L/min.)	Detection limit (µg/L)	Blank Contribution (%)
Ca	422.7	6.0	0.5	2.0	4.0	0.9
Cd	228.8	4.0	0.3	1.8	4.0	0.9
Co	240.7	6.0	0.2	2.2	5.0	0.6
Cr	357.9	5.0	0.5	2.6	6.0	0.7
Cu	324.8	3.0	0.5	1.8	4.0	0.4
Fe	248.3	8.0	0.2	2.0	6.0	0.8
Mg	285.2	4.0	0.5	1.6	1.0	1.3
Mn	279.5	5.0	0.4	1.9	3.0	0.3
Pb	217.0	7.0	0.3	1.8	10.0	1.2
Zn	213.9	4.0	0.5	2.0	2.0	1.1

2.4. Analyses of the metals. In the present study, quantitative measurements of selected essential and toxic metals including Ca, Mg, Fe, Zn, Cu, Co, Mn, Cr, Cd and Pb were performed on Flame Atomic Absorption Spectrophotometer (Shimadzu AA-670, Japan). It has the capacity to operate automatically in background compensation mode, thus correcting fluctuations in the observed signal arising from factors other than the sample. Other salient features of the equipment included automatic operational conditions; recording of the data including the calibration curve and precise data processing function to ensure high analytical precision and accuracy. Lamp position, detector gain and beam balancing are adjusted automatically with convenience to exclude the deviant data in repeated analytical modes. Various analytical parameters related to the instrument such as wavelength, hollow cathode lamp current, slit width, flame type and fuel/oxidant flow rates were optimized for the analysis of each metal independently

as shown in Table 1. The metal data were processed for statistical evaluation including both univariate and multivariate methods.

3. RESULTS AND DISCUSSION

3.1. Demographic characteristics. The demographic data of osteoarthritis patients and healthy subjects is shown in Table 2. The patients and healthy subjects were closely matched for age, gender, habitat, food habits and smoking habits. Among the selected subjects, 52% of the patients and 56% of healthy subjects were females. Most of the healthy subjects and the patients (67% and 64%, respectively) were the residents of urban areas. The patients with vegetarian food habits constituted 36% of the subjects while on the other hand 32% of the healthy subjects were predominantly vegetarians. Smoking based assortment of the subjects revealed that majority of the patients (78%) healthy subjects (82%) were not addicted of smoking on regular basis. Overall, the patients and healthy subjects demonstrated almost comparable demographic characteristics.

Table 2. Characteristics of the Subjects.

Characteristics	Patients	Controls
n	77	82
Age (years)		
Range	23-74	21-75
Average	45.45	43.61
Gender		
Male	37 (48%)	36 (44%)
Female	40 (52%)	46 (56%)
Habitat		
Urban	49 (64%)	55 (67%)
Rural	28 (36%)	27 (33%)
Food Habit		
Vegetarian	28 (36%)	26 (32%)
Non-Vegetarian	49 (64%)	56 (68%)
Smoking habit		
Smoking	17 (22%)	15 (18%)
Non-smoking	60 (78%)	67 (82%)

3.2. Distribution of selected metals in the blood. Basic statistical parameters related to the distribution of selected essential and toxic metal levels in the blood of osteoarthritis patients are shown in Table 3. Most of the metals exhibited broad range and large variations in their minimum and maximum concentrations. On the average basis, comparatively higher levels were found for Fe (239.7 µg/g), Ca (159.1 µg/g) and Mg (29.02 µg/g), followed by relatively lower levels of Pb (5.729 µg/g), Zn (3.735 µg/g), Co (2.918 µg/g), Cr (2.198 µg/g) and Cu (1.576 µg/g). However, lowest average concentrations were noted for Mn and Cd at 0.550 and 0.473 µg/g, respectively. On the average basis, decreasing trend of metal levels in the blood of OA patients revealed following order: Fe > Ca > Mg > Pb > Zn > Co > Cr > Cu > Mn > Cd. The major constituents of the blood (Fe, Ca and Mg) are involved in vital metabolic processes [37, 38]. Most of the metals exhibited random distribution pattern as manifested by considerably large SD and SE values as well as markedly dissimilar mean and median levels. Comparatively higher dispersion in the concentrations was noted for Fe, Ca, Mg and Pb as indicated by elevated SD and SE values. Some of the metals (Co, Cr, Cu and Zn) showed relatively lower dispersion as indicated by moderate SD and SE values, however, least dispersion was found for Cd and Mn in terms of the lowest SD and SE values. Large skewness values for Mn, Zn and Cu showed their predominantly asymmetric distribution, while moderate skewness values noted for Ca, Cd and Pb indicated relatively lower lop-sidedness in the distribution of these metals in the blood of OA patients. Nonetheless, comparatively symmetric distribution was observed for Co, Cr, Fe and Mg as evidenced by the lowest values of skewness and kurtosis. The quartile distribution (Figure 1) showed that Ca, Fe and Mg displayed very narrow distribution as specified by small variations in upper/lower quartiles, whereas considerable variations were observed for Cd, Pb and Zn with more or less symmetrical upper and lower quartiles. Nevertheless, large and asymmetrical variations in the quartile distribution were found for Co, Cr, Cu and Mn in the blood of OA patients.

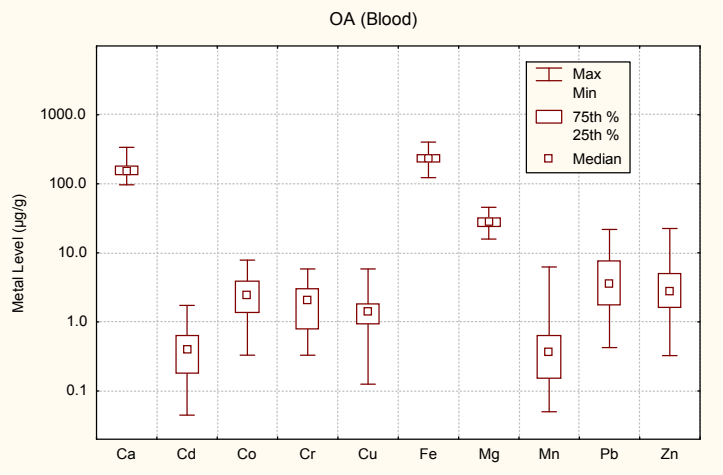

Figure 1. Quartile distribution for selected metal levels (µg/g, wet weight) in the blood of osteoarthritis (OA) patients.

Distribution of the selected metal levels in the blood of healthy subjects as revealed by basic statistical parameters is also shown in Table 3. Most of the metals exhibited variations by several orders of magnitude as shown by the minimum and maximum concentrations. Comparatively higher mean levels were noted for Fe (305.4 µg/g) and Ca (169.5 µg/g), followed by relatively lower levels of Mg (41.66 µg/g), Zn (8.934 µg/g), Pb (4.030 µg/g), Cu (1.854 µg/g), Co (1.564 µg/g) and Cr (1.262 µg/g), while lowest average levels were found for Mn and Cd. Mean concentrations of the metals in blood of normal donors showed following decreasing order: Fe > Ca > Mg > Zn > Pb > Cu > Co > Cr > Mn > Cd. Some of the metals (Ca, Fe, Mg, Zn, Pb and Cu) exhibited predominantly non-Gaussian distribution as revealed by corresponding higher SD and SE values, while rest of the metals showed relatively lower dispersion in their concentrations. Skewness and kurtosis values indicated relatively symmetrical variations for Ca, Fe and Mn while the remaining metals showed considerably asymmetrical variations in the blood of healthy donors. Quartile distribution of the metals in the blood of the healthy subjects is shown as box-whisker plot in Figure 2. Among the metals in the blood of healthy subjects, Ca, Co, Cr, Cu and Fe showed relatively broad distribution with significantly divergent upper and lower quartiles. Nevertheless, Cd and Mn showed very narrow distribution evidenced by overlapping of upper and lower quartiles over the median levels. Among the selected metals, Mg showed significant asymmetry towards lower quartile. Overall, the quartile distribution of most of the metals in

the blood of healthy subjects was considerably divergent than the patients.

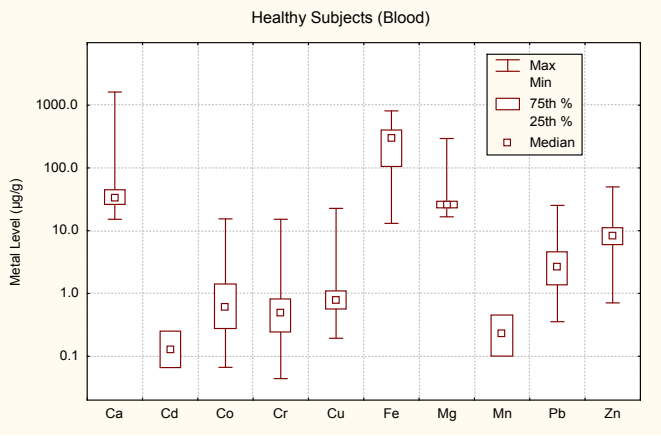

Figure 2. Quartile distribution for selected metal levels (µg/g, wet weight) in the blood of healthy subjects.

Comparative evaluation of the metals levels in the blood of the patients and controls revealed that majority of the metals including Cd, Co, Cr, Mn and Pb exhibited relatively higher contributions in the blood of OA patients than healthy subjects, while healthy donors exhibited fairly higher average levels of Cu, Fe, Mg and Zn than the patients. Relatively higher concentrations of the toxic/trace metals in the blood of OA patients indicated active role of these metals in the metabolism during osteoarthritis. Nevertheless, mean levels of Ca were found almost comparable in both study groups, which suggested that blood levels of Ca had little effects on progress of OA disease. Higher levels of Pb in the blood are associated with increased occurrence and severity of OA indicating contribution of environmental factor for the onset and progress of the disease [39, 40]. Another study related to the distribution of metals in arthritis patients in comparison to healthy controls indicated higher contribution of Mn and lower contribution of Zn in the serum of the patients compared with healthy subjects [41]. Overall, the proportional variations of toxic and essential metals in the blood of the OA patients were significantly diverse than healthy donors.

Table 3. Statistical distribution parameters for selected metal concentrations (µg/g, wet weight) in the blood of osteoarthritis patients and healthy subjects.

	Osteoarthritis Patients								Healthy Subjects							
	Min	Max	Mean	Median	SD	SE	Skew.	Kurt.	Min	Max	Mean	Median	SD	SE	Skew.	Kurt.
Ca	96.58	337.4	159.1	153.6	41.41	5.978	1.672	6.179	15.21	1398	169.5	33.99	388.8	52.42	2.878	7.355
Cd	0.045	1.734	0.473	0.401	0.382	0.055	1.516	2.338	0.014	2.785	0.259	0.130	0.468	0.061	4.417	20.71
Co	0.330	7.845	2.918	2.452	1.951	0.282	0.926	0.195	0.067	15.44	1.564	0.620	2.758	0.359	3.526	13.71
Cr	0.330	5.841	2.198	2.057	1.524	0.225	0.804	0.029	0.044	15.21	1.262	0.495	2.436	0.326	4.118	20.13
Cu	0.125	5.845	1.576	1.436	1.015	0.147	2.161	6.653	0.193	22.71	1.854	0.784	4.142	0.518	3.988	15.37
Fe	122.8	401.4	239.7	235.1	55.08	7.950	0.470	1.375	13.11	811.6	305.4	302.4	199.5	24.74	0.524	-0.398
Mg	15.84	45.65	29.02	28.82	6.678	0.964	0.343	0.145	16.64	294.3	41.66	26.10	59.00	7.318	3.768	13.20
Mn	0.050	6.246	0.550	0.374	0.909	0.133	5.584	35.00	0.009	1.803	0.344	0.227	0.377	0.051	2.354	6.397
Pb	0.425	21.82	5.729	3.557	6.012	0.886	1.587	1.478	0.355	25.32	4.030	2.712	4.678	0.655	3.060	10.88
Zn	0.325	22.52	3.735	2.759	3.535	0.510	3.369	16.44	0.708	49.86	8.934	8.484	6.997	0.919	3.854	20.90

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

		Ca	Cd	Co	Cr	Cu	Fe	Mg	Mn	Pb	Zn
Osteoarthritis Patients	Ca	1									
	Cd	**0.311**	1								
	Co	0.065	0.276	1							
	Cr	0.115	0.228	0.265	1						
	Cu	0.171	-0.030	-0.103	**0.408**	1					
	Fe	0.016	0.195	0.009	**0.351**	**0.367**	1				
	Mg	-0.055	-0.112	0.247	0.140	-0.209	-0.008	1			
	Mn	-0.177	-0.100	-0.024	0.242	**0.604**	0.192	0.016	1		
	Pb	-0.120	-0.043	-0.038	0.058	**0.322**	0.068	-0.239	**0.346**	1	
	Zn	-0.055	-0.116	0.074	-0.009	0.203	0.118	-0.084	-0.114	0.233	1
Healthy Subjects	Ca	1									
	Cd	**0.683**	1								
	Co	0.146	**0.717**	1							
	Cr	**0.749**	**0.543**	**0.613**	1						
	Cu	**0.742**	**0.705**	**0.597**	**0.811**	1					
	Fe	**-0.322**	-0.246	0.034	-0.173	-0.274	1				
	Mg	**0.822**	**0.755**	**0.685**	**0.883**	**0.953**	-0.297	1			
	Mn	**0.484**	0.268	**0.422**	**0.320**	0.206	0.002	0.277	1		
	Pb	0.024	0.136	0.064	-0.018	0.107	**-0.364**	0.178	-0.045	1	
	Zn	-0.249	-0.222	-0.138	-0.258	**0.304**	0.023	-0.241	-0.289	-0.029	1

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

3.3. Correlation study of selected metals in the blood. The metal-to-metal correlations in the blood of OA patients (Table 4) showed significant and positive correlations among Mn-Cu (r = 0.604), Cu-Cr (r = 0.408), Fe-Cu (r = 0.367), Fe-Cr (r = 0.351), Pb-Mn (r = 0.346), Pb-Cu (r = 0.322) and Ca-Cd (r = 0.311). The correlation study revealed strong associations of Cu with Mn, Cr, Fe and Pb which indicated their dependency in occurrence on each other. Similarly significant relationship of Ca and Cd indicated their role in biological processes. The correlation study indicated some mutual associations among the essential and toxic metals, such as; Ca with Cd, Fe with Cr, Cu with Pb. The toxic metals (Cd and Pb) can interact with essential metals; Pb interacts with Ca to affect negatively cognitive development [42].

The correlation coefficient matrix for selected essential and toxic metals in the blood of healthy subjects is also shown in Table 4. Very strong positive correlations were noted between Cu-Mg (r = 0.953), Mg-Cr (r = 0.883), Ca-Mg (r = 0.822), Cr-Cu (r = 0.811), Cd-Mg (r = 0.755), Cr-Ca (r = 0.749), Cu-Ca (r = 0.742), Co-Cd (r = 0.717), Cu-Cd (r = 0.705), Mg-Co (r = 0.685), Cd-Ca (r = 0.683), Co-Cr (r = 0.613), Cu-Co (r = 0.597) and Cd-Cr (r = 0.543). Some negative relationships were found for Pb-Fe (r = -0.364) and Ca-Fe (r = -0.322). In the case of healthy subjects, the correlation study indicated an apparently common origin of essential metals (e.g. Ca and Mg) while the toxic/trace metals (including Cd, Co, Cr and Cu) were mutually correlated indicating their common origin. Similar trends were observed by another study involving trace metals measurement in the blood of healthy subjects in comparison with ovary cancer patients [43]. Overall, the correlation pattern of toxic and essential metals in the blood of healthy subjects remained evidently diverse compared with the patients; it may be attributed to the disproportions of the nutrients and trace metals in the case of OA patients.

3.4. Multivariate analysis of selected metals in the blood. Another important aspect of the present study is multivariate analysis of the essential and toxic metals in the blood of OA patients and healthy subjects. The principle component analysis (PCA) of the metal levels in blood of the patients extracted by varimax normalized rotation on the data-set are shown in Table 5, whereas the corresponding CA based on Ward's method is shown in Figure 3. In the case of OA patients, four PCs were extracted with eigen values greater than 1, cumulatively explaining more than 65% of the total variance. The cluster analysis (CA) of the metal levels in the blood of OA patients demonstrated three strong clusters; Zn-Pb, Mn-Cu-Fe-Cr and Co-Cd-Ca-Mg. Similarly, PC 1 showed higher loading for Cr, Cu, Fe and Mn with a strong mutual cluster of these metals in CA, manifesting the common origin of these metals which may be traced in anthropogenic contributions alongside the dietary intake. Likewise, PC 2 revealed elevated loading for Ca and Cd, which were mostly contributed by dietary habits and environmental pollutants. PC 3 exhibited significant loadings for Co, Cr and Mg, which were mainly regulated by internal metabolism. Lastly, PC 4 showed elevated loadings for Pb and Zn, well supported by CA. In most of the previous studies, these metals were ascribed to anthropogenic pollutants, particularly the activities related to the combustion of fuels; however Pb and Zn are also emitted in fly-ash from coal fired power plants [44].

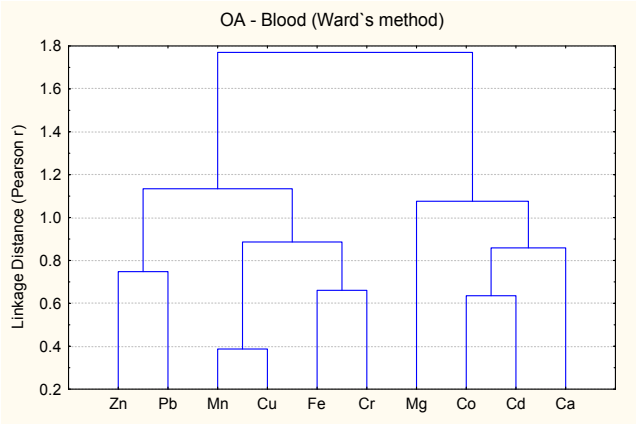

Figure 3. Cluster analysis of selected metals in the blood of osteoarthritis (OA) patients.

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

	Osteoarthritis Patients				Healthy Subjects			
	PC 1	PC 2	PC 3	PC 4	PC 1	PC 2	PC 3	PC 4
Eigen value	2.318	1.821	1.338	1.084	3.796	1.787	1.395	1.221
Total Variance (%)	23.18	18.21	13.38	10.84	37.96	17.87	13.95	12.21
Cumulative eigen value	2.318	4.139	5.476	6.560	3.796	5.583	6.978	8.199
Cumulative Variance (%)	23.18	41.39	54.76	65.60	37.96	55.83	69.78	81.99
Ca	0.024	0.757	-0.104	-0.128	0.972	-0.084	0.043	0.099
Cd	0.026	0.772	0.227	0.000	0.200	-0.072	0.790	-0.201
Co	-0.038	0.246	0.786	0.123	0.064	-0.081	0.151	0.922
Cr	0.576	0.232	0.526	0.043	0.670	-0.001	0.142	-0.659
Cu	0.856	0.066	-0.187	0.185	-0.159	0.049	0.804	0.343
Fe	0.529	0.234	0.116	0.151	-0.219	0.808	-0.306	-0.087
Mg	-0.068	-0.301	0.694	-0.308	0.957	-0.045	-0.127	0.085
Mn	0.813	-0.309	-0.021	-0.148	0.746	-0.037	0.136	-0.543
Pb	0.405	-0.168	-0.200	0.518	0.035	0.912	0.198	0.020
Zn	0.009	-0.044	0.051	0.903	0.561	-0.117	0.145	-0.332

The PCA of essential and toxic metal levels in the blood of healthy subjects extracted by varimax normalized rotation on the data-set are also shown in Table 5, while the corresponding CA is portrayed in Figure 4. In the case of healthy subjects, the PCA yielded four PCs of the metals with eigen values higher than 1, commutatively exhibiting more than 81% of the total variance of data. The CA of the metal data in the blood of healthy subjects revealed very strong clusters of Zn-Fe; Pb-Cu-Cd; Mn-Cr-Co and Mg-Ca. PC 1 showed higher loadings for Ca, Mg, Cr and Mn; these metals were mostly contributed by the dietary intakes with some contributions from external environmental conditions. Similarly, PC 2 showed elevated loadings for Fe and Pb which were mainly associated with anthropogenic sources alongside natural contributions. Third PC showed elevated loadings of Cd and Cu, while PC 4 exhibited rather higher loadings for Co-Cu in the blood of healthy subjects. These two PCs were mostly originating from anthropogenic sources, especially industrial waste/effluents. Overall, the PCA and CA findings evidenced that the origin and apportionment of the metals in the blood of OA patients and healthy subjects were significantly dissimilar with diverse contributing factors, which may be ascribed to the altered metabolism due to the disease. In the case of OA patients, toxic metals were strongly associated with some essential metals (e.g., Cd showed interference with Ca and Mg, Cr and Cu with Fe, and Pb with Zn). In contrast, the healthy subjects showed typically separate groups of the essential and toxic metals. These associations indicated that the metabolism of essential metals was significantly affected and/or altered by toxic metals in the case of

patients. Based on these deliberations, it can be assumed that PCA/CA may be used as a diagnostic tool in clinical studies although it required further support/justification by detailed studies comprising of larger population segments.

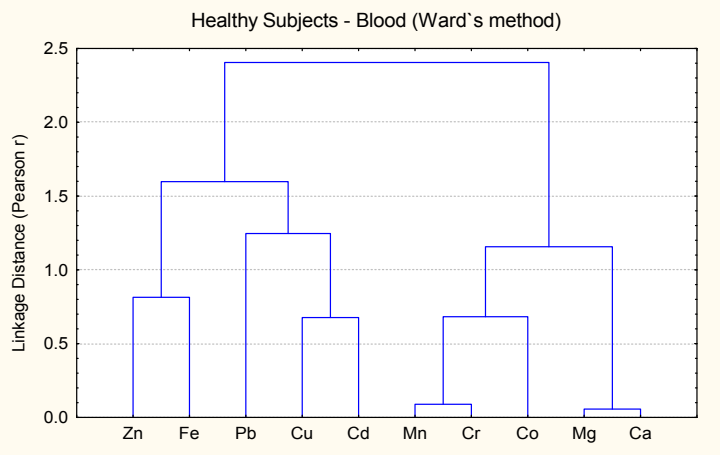

Figure 4. Cluster analysis of selected metals in the blood of healthy subjects.

3.5. Gender-based comparison of the metal levels in OA patients and healthy subjects. The gender-based variations of average metals levels (± SE) in the blood of OA patients in comparison with healthy subjects (Figure 5) revealed that most of the metals were relatively higher in the blood of male patients compared with the female patients; this may have some relationship with the possibility of metabolism in males/females as it has been reported that there is significant effect of gender on the distribution of micronutrients and trace metals in the blood of the donors [45]. The mean concentrations of Co, Cu, Fe, Mn, Pb and Zn were found to be significantly higher in the male patients, while Cd and Cr were noticeably higher in the blood of female patients; however Ca and Mg have comparable average levels in both female and male patients. In contrast to the patients, male and female controls showed an entirely different pattern; except Pb and Zn all the metals showed higher levels in the blood of male healthy subjects than female donors. Similarly, comparison of the metal levels in male patients and male controls showed significant differences; mean levels of Cd, Co, Mn and Pb were considerably higher in the blood of male patients while the essential metals (such as Ca, Cu, Fe, Mg and Zn) exhibited higher average levels in the male healthy subjects. A recent study reported considerably lower levels of Fe in OA patients compared with the healthy subjects [46]. The female patients showed higher levels of Ca, Cd, Co, Cr, Cu, Mg and Mn than the female controls which showed relatively higher average levels of Fe, Pb and Zn. Among these metals, Fe and Zn play very important role in metabolism of bone; a previous study reported positive correlation of Zn with Fe in the synovial fluid [46]. It has been reported that Zn, Cu, and Fe levels may change in the course of inflammatory process [47].

3.6. Habitat-based comparison of the metal levels in OA patients and healthy subjects. Comparative average concentrations (± SE) of the metals in the blood of OA patients from rural and urban residential premises in comparison with counterpart healthy subjects are shown in Figure 6. It has been reported that environmental factors may contribute to the onset and progression of autoimmune disease [48]. Average concentrations of Cd, Cr, Fe, Mg and Pb were found almost comparable in both urban/rural patients; however the average levels of Ca and Cu were found elevated in the blood of the patients from rural areas while Co and Zn were considerably higher in the blood of the patients from urban areas. In the case of healthy subjects, mean levels of Ca, Cd, Co, Cr, Cu, Mg and Mn were found significantly higher in the rural subjects compared to the urban subjects, which showed relatively elevated average levels of Fe, Pb and Zn. The patients living in urban residential areas showed considerably higher concentrations of Ca, Cd, Co, Cr, Cu, Mg, Mn and Pb in comparison with the urban controls whereas rural patients exhibited elevated concentrations of Mn and Pb in comparison with their counterpart rural controls. Many kinds of abnormalities in the distribution of trace metals have been diagnosed in inflammatory diseases including osteoarthritis since long but interpretation of these changes have not been explained yet [49, 50, 51]. In general, comparative variations of the essential and toxic metals in the blood of the patient and healthy subjects from rural and urban localities showed considerable disparities indicating a possible mode of metal imbalances due to the environmental factors.

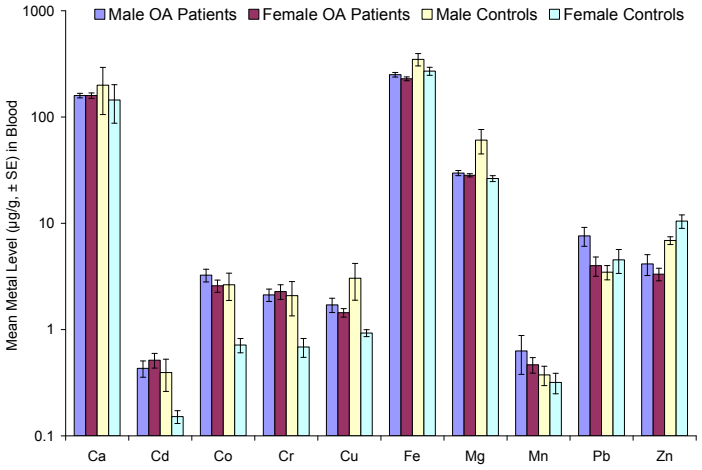

Figure 5. Gender-based comparison of average metal levels (µg/g ± SE) in the blood of osteoarthritis (OA) patients and healthy subjects/controls.

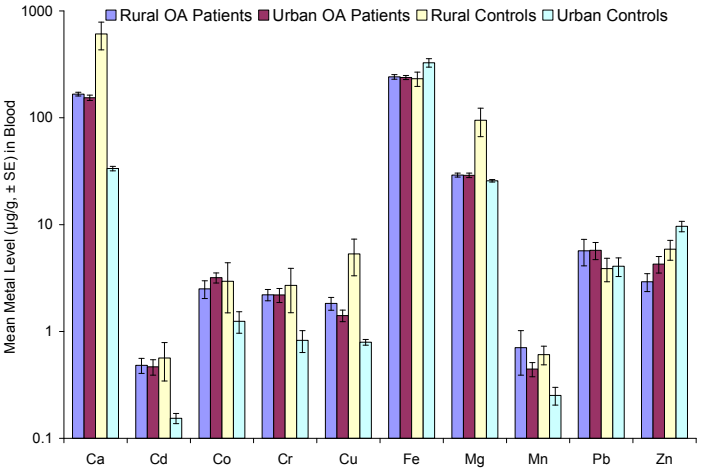

Figure 6. Habitat-based comparison of average metal levels (µg/g ± SE) in the blood of osteoarthritis (OA) patients and healthy subjects/controls.

3.7. Diet-based comparison of the metal levels in OA patients and healthy subjects. Average levels of selected metals in the blood of OA patients and controls with vegetarian/non-vegetarian food habits are shown in Figure 7. Former research studies have shown that food habits have an important contribution in autoimmune diseases; fasting and subsequent controlled vegetarian diet is shown to lessen the activity of the disease [52].

Among the selected metals, Cu, Pb and Zn showed elevated levels in the blood of vegetarian patients, while Cd, Co, Cr and Mn were found higher in the non-vegetarian patients. However, Ca, Fe and Mg levels manifested almost comparable contributions in both groups showing that vegetarian and non-vegetarian food habits did not have any effect on these metals. In the case of healthy subjects, Mn was found elevated in the blood of vegetarian controls while average levels of the other metals were noted relatively higher in the non-vegetarian controls. Similarly, comparison of the average metal levels in the blood of vegetarian patients with vegetarian controls showed that mean levels of Mg and Mn were noted almost comparable in both groups, while Fe and Zn were found significantly higher in the vegetarian controls. Nonetheless, Ca, Cd, Co, Cr, Cu and Pb contents were comparatively higher in the patients with vegetarian food habits. Comparison of the average metal levels in non-vegetarian patients with counterpart non-vegetarian controls indicated that Cd, Co, Cr, Mn and Pb levels were significantly higher in non-vegetarian patients while Cu, Fe, Mg and Zn levels were noted elevated in the non-vegetarian controls. It has been reported that higher levels of red meat and protein consumption found correlated with greater risk of autoimmune inflammatory diseases [53], however some other studies suggested no proven relationship of red meat and protein consumption with the risk of arthritis [54].

3.8. Smoking-based comparison of the metal levels in OA patients and healthy subjects. Average metal levels in the blood of OA patients with smoking/non-smoking habits in comparison with similar healthy subjects are shown in Figure 8. Studies related to the epidemiology have indicated the association of various types of arthritis with smoking habits or excessive exposure to the tobacco [55-61]. It has been suggested that the immune system of the body might be activated due to exposure of respiratory organs to the cigarette smoke. Mean concentrations of Co, Cr, Cu, Fe, Mg, Mn and Pb were found to be comparatively higher in the blood of patients with smoking habits, while in comparison non-smoking patients depicted higher levels of Ca and Cd in their blood. However, Zn exhibited almost comparable contribution in both groups.

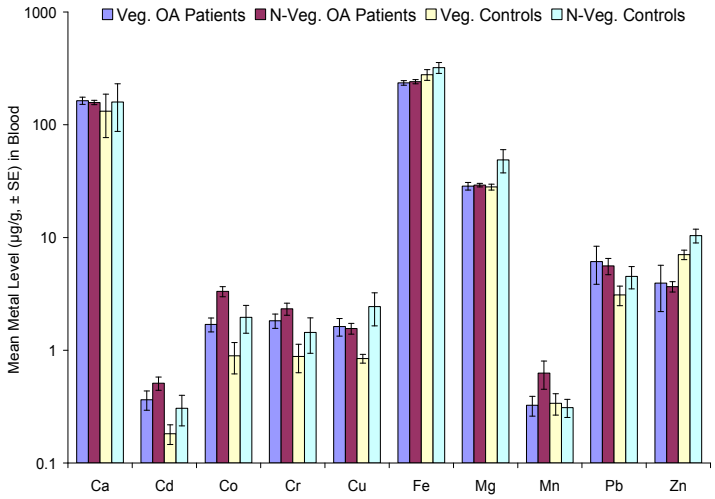

Figure 7. Diet-based comparison of average metal levels (µg/g ± SE) in the blood of osteoarthritis (OA) patients and healthy subjects/controls.

It has been reported that smoking may contribute in the accumulation of toxic metals; mean contents of Co, Cr and Cd were found to be significantly higher in the smoking controls compared to the non-smoking controls. Similarly, mean concentrations of Co, Mn and Pb were found elevated in the blood of smoking patients in comparison with smoking healthy subjects. This pattern indicated that the higher concentrations of Co, Mn and Pb in the blood of OA patients may contribute towards the onset and progression of the disease. The risk of arthritis is believed to be directly related to smoking; normally it increases with continued smoking [62]. Overall, comparative assessment of the metals in the blood of smoking and non-smoking subjects showed substantial disproportions indicating the metal imbalances due to smoking habits.

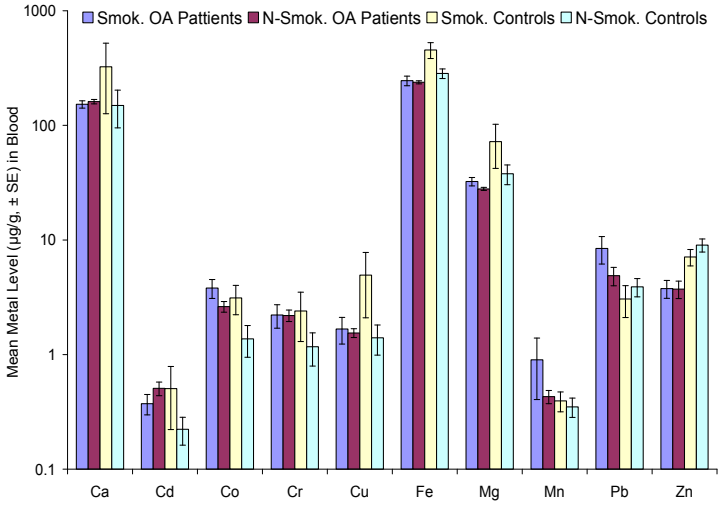

Figure 8. Smoking-based comparison of average metal levels (µg/g ± SE) in the blood of osteoarthritis (OA) patients and healthy subjects/controls.

3.9. Variations of the metal levels with age of the patients and controls. Variations in the metal levels with the age of OA patients and controls revealed that the concentrations of Cr and Cu in the blood exhibited significant diverse relationships, while other metals demonstrated insignificant associations with the age. Variations of Cr levels in the blood of the patients and controls with age are shown in Figure 9. The results indicated that Cr levels exhibited increasing trend with the age of the patients but on contrary it showed decreasing trend with the age of healthy subjects. Elevated Cr levels were associated with higher burden of the free radicals [14, 63]. It takes part in redox reactions by converting itself to various oxidation states; thus it may act as cytotoxcin, carcinogenic and genotoxcin. Higher concentrations of Cr and its long term exposure may affect the immune system; however its mechanism is not clearly understood [64]. Similarly, comparative variations in the concentrations of Cu in the blood of OA patients and healthy donors with their age are illustrated in Figure 10. Proportional appraisal revealed that Cu levels exhibited a significant build-up in the blood of the patients with increasing age, however it exhibited depleting pattern in the blood of the healthy subjects. Relatively higher concentrations of Cu in the blood of the patients with age are due to the fact that during inflammation processes in autoimmune diseases ceruloplasmin is released in the blood; it increases the concentration of Cu in the blood of the patients. Elevated concentrations of Cu were also linked with the excessive formation of the free radicals and promotion of the autoimmune diseases. These findings are in agreement with the previous studies [46, 65].

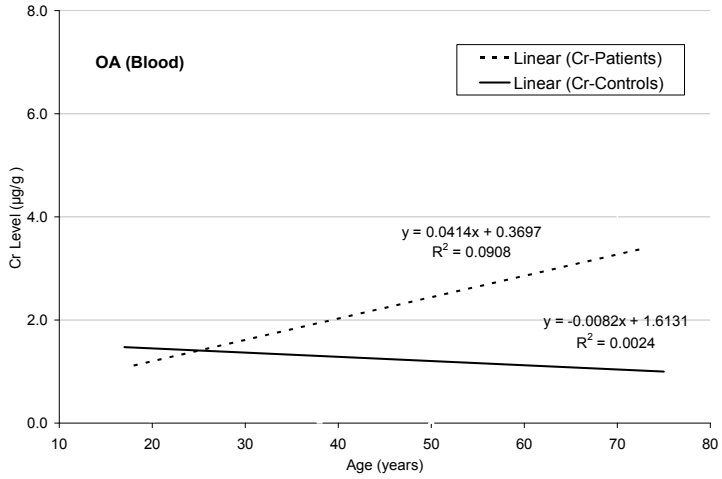

Figure 9. Comparative variations of Cr levels in the blood with the age of OA patients and healthy subjects/controls.

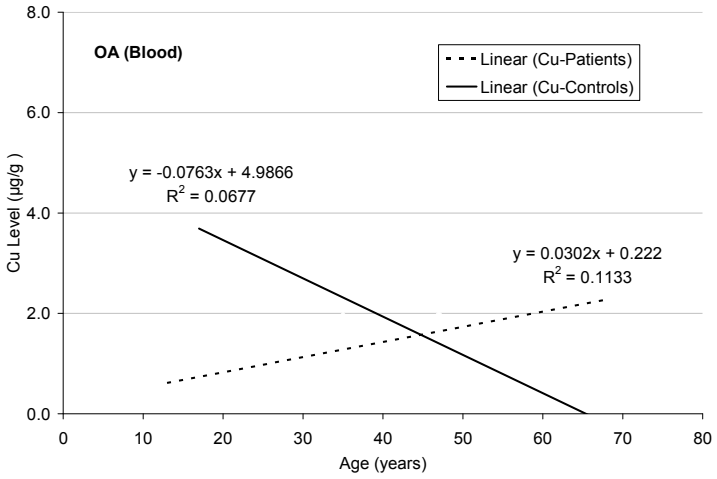

Figure 10. Comparative variations of Cu levels in the blood with the age of OA patients and healthy subjects/controls.

4. CONCLUSIONS

The present study showed divergent distribution of essential and toxic metals in the blood of OA patients and healthy subjects. Significantly higher average concentrations of Cd, Co, Cr, Mn and Pb were noted in the blood of the patients in comparison with the controls which in turn showed higher concentrations of Cu, Fe, Mg and Zn. Correlation study showed diverse associations among the essential and toxic metals in the patients and controls. PCA and CA revealed mutual clusters/loadings for Co-Cd-Ca-Mg, Mn-Cu-Fe-Cr and Pb-Zn in the patients while healthy subjects showed common clusters/loadings for Ca-Mg, Fe-Zn, Mn-Cr-Co and Cd-Cu-Pb. Significant variations in the metal levels were noted in the patients and healthy subjects depending on their gender, habitat, food habits and smoking habits. Significant accumulation of Cr and Cu with the age was observed in the patients while the controls showed depleting tendency of these metals with their age.

5. REFERENCES

[1] Hinton, R., Moody, R. L., Davis, A. W., Thomas, S. F., Osteoarthritis: diagnosis and therapeutic considerations, *American Family Physician,* 65, 841-848, **2002.**

[2] Galois, L., Etienne, S., Grossin, L., Watrin-Pinzano, A., Cournil-Henrionnet, C., Loeuille, D., Netter, P., Mainard, D., Gillet, P., Dose-response relationship for exercise on severity of experimental osteoarthritis in rats: a pilot study, *Osteoarthritis and Cartilage*, 12, 779-786, **2004**.

[3] Shivakumar, B., Licofelone: A Novel Non-Steriodal Anti-Inflammatory Drug (NSAID) in Arthritis, *Iranian Journal of Pharmacology & Therapeutics*, 10, 82-91, **2012.**

[4] Bove, S. E., Calcaterra, S. L., Brooker, R. M., Huber, C. M., Guzman, R. E., Juneau, P. L., Schrier, D. J., Kilgore, K. S., Weight bearing as a measure of disease progression and efficacy of anti-inflammatory compounds in a model of monosodium iodoacetate-induced osteoarthritis, *Osteoarthritis and Cartilage,* 11, 821-830, **2003.**

[5] McColl, G. J., Pharmacological therapies for the treatment of osteoarthritis, *The Medical Journal of Australia,* 175, S108-S111, **2001.**

[6] Bouchgua, M., Alexander, K., d'Anjou, M. A., Girard, C. A., Carmel, E. N., Beauchamp, G., Richard, H., Laverty, S., Use of routine clinical multimodality imaging in a rabbit model of osteoarthritis-part I, *Osteoarthritis and Cartilage,* 17, 188-196, **2009.**

[7] Morenko, B. J., Bove, S. E., Chen, L., Guzman, R. E., Juneau, P., Bocan, T., Peter, G. K., Arora, R., Kilgore, K. S., In vivo micro computed tomography of subchondral bone in the rat after intra-articular administration of monosodium iodoacetate, *Journal of the American Association for Laboratory Animal Science,* 43, 39-43, **2004.**

[8] Gutteridge, J. and B. Halliwell, Antioxidants: molecules, medicines, and myths, *Biochemical and Biophysical Research Communications,* 393, 561-564. **2010.**

[9] Hindmarsh, J. T., Abernethy, C. O., Peters, G. R., McCurdy, R. F., *Environmental Aspects of Arsenic Toxicity. Heavy Metals in the Environment*, Marcel Dekker, Inc, 217-229, **2002**

[10] Davies, C. M., Guilak, F., Weinberg, J. B., Fermor, B., Reactive nitrogen and oxygen species in interleukin-1-mediated DNA damage associated with osteoarthritis, *Osteoarthritis and Cartilage,* 16, 624-630, **2008.**

[11] Moini, H., Packer, L., Saris, N. E. L., Antioxidant and prooxidant activities of α-lipoic acid and dihydrolipoic acid, *Toxicology and Applied Pharmacology,* 182, 84-90, **2002.**

[12] Afonso, V., Champy, R., Mitrovic, D., Collin, P., Lomri, A., Reactive oxygen species and superoxide dismutases: role in joint diseases, *Joint Bone Spine,* 74, 324-329, **2007.**

[13] Henrotin, Y. E., Bruckner, P., Pujol, J. P., The role of reactive oxygen species in homeostasis and degradation of cartilage, *Osteoarthritis and Cartilage,* 11, 747-755, **2003.**

[14] Pham-Huy, L. A., He, H., Pham-Huy, C., Free radicals, antioxidants in disease and health, *International Journal of Biomedical Science,* 4, 89-96, **2008.**

[15] Hashmi, G. M., Shah, M. H., Comparative Assessment of Essential and Toxic Metals in the Blood of Rheumatoid Arthritis Patients and Healthy Subjects, *Biological Trace Element Research,* 146, 13-22, **2012.**

[16] Volpe, M. G., La Cara, F., Volpe, F., De Mattia, A., Serino, V., Petitto, F., Zavalloni, C., Limone, F., Pellecchia, R., De Prisco, P. P., Di Stasio, M., Heavy metal uptake in the enological food chain. *Food Chemistry,* 117, 553-560, **2009.**

[17] Hashmi, G. M. and M. H. Shah, Comparative evaluation of trace elements in the scalp hair of arthritis patients and healthy donors, *Toxicological & Environmental Chemistry,* 93, 2123-2134, **2011.**

[18] Scheckel, K. G., Chaney, R. L., Basta, N. T., Ryan, J. A., Advances in assessing bioavailability of metal (loid) s in contaminated soils, *Advances in Agronomy,* 104, 1-52, **2009.**

[19] Zhang, G. Z., Li, M. H., Yang, S. O. N. G., Ting, L. I. U., Shen, G. A. O. and Ying, S. U. N., Effects of micronutrients on oxidative stress and islet function in type 1 Diabetes Mellitus rats: Relationships to Th2 type cytokines, interleukin-4, and interluekin-10, *Chemical Research in Chinese Universities,* 23, 701-704, **2007.**

[20] Campbell, J., Lifestyle, minerals and health, *Medical Hypotheses,* 57, 521-531, **2001.**

[21] Holick, M. F., Chen T. C., Vitamin D deficiency: a worldwide problem with health consequences, *The American Journal of Clinical Nutrition,* 87, 1080S-1086S, **2008.**

[22] Da Silva, J. F., Williams R. J. P., *The Biological Chemistry of the Elements: The Inorganic Chemistry of Life*, Oxford University Press**. 2001**

[23] Andreini, C., Bertini, I., Cavallaro, G., Holliday, G. L., Thornton, J. M., Metal ions in biological catalysis: from enzyme databases to general principles, *Journal of Biological Inorganic Chemistry,* 13, 1205-1218, **2008.**
[24] Frestedt, J. L., Kuskowski, M. A., Zenk, J. L., A natural seaweed derived mineral supplement (Aquamin F) for knee osteoarthritis: a randomised, placebo controlled pilot study, *Nutritional Journal,* 8, 7, **2009.**
[25] King, D. E., Mainous III, A. G., Geesey, M. E., Woolson, R. F., Dietary magnesium and C-reactive protein levels, *Journal of the American College of Nutrition,* 24, 166-171, **2005.**
[26] Frestedt, J. L., Walsh, M., Kuskowski, M. A., Zenk, J. L., A natural mineral supplement provides relief from knee osteoarthritis symptoms: a randomized controlled pilot trial, *Nutritional Journal,* 7, p1, **2008**.
[27] Salgueiro, M. J., Zubillaga, M. B., Lysionek, A. E., Caro, R. A., Weill, R., Boccio, J. R., The role of zinc in the growth and development of children, *Nutrition,* 18, 510-519, **2002.**
[28] Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage, *Current Topics in Medicinal Chemistry,* 1, 529-539, **2001.**
[29] Akiibinu, M. O., Ogundahunsi, A. O., Kareem, O. I., Adesiyan, A. A., Idonije, B. O., Adeniyi, F. A. A., Trace metals and oxidative metabolic changes in malignant prostate cancer patients, *African Journal of Biochemistry Research,* 5, 102-105, **2011.**
[30] Tan, C., Chen, H., Zhu, W., Application of boosting classification and regression to modeling the relationships between trace elements and diseases, *Biological Trace Element Research,* 134, 146-159, **2010.**
[31] Vural, H., Uzun, K., Uz, E., Kocyigit, A., Cigli, A., Akyol, O., Concentrations of copper, zinc and various elements in serum of patients with bronchial asthma, *Journal of Trace Elements in Medicine and Biology,* 14, 88-91, **2000.**
[32] Yaman, M., Atici, D., Bakirdere, S., Akdeniz, I., Comparison of trace metal concentrations in malign and benign human prostate, *Journal of Medicinal Chemistry,* 48, 630-634, **2005.**
[33] Krachler, M., Rossipal, E., Micetic-Turk, D., Concentrations of trace elements in sera of newborns, young infants, and adults, *Biological Trace Element Research,* 68, 121-135, **1999.**
[34] Rowley, B., Monestier, M., Mechanisms of heavy metal-induced autoimmunity, *Molecular Immunology,* 42, 833-838, **2005.**
[35] Hees, T., Wenclawiak, B., Lustig, S., Schramel, P., Schwarzer, M., Schuster, M., Verstraete, D., Dams, R., Helmers, E., Distribution of platinum group elements (Pt, Pd, Rh) in environmental and clinical matrices: composition, analytical techniques and scientific outlook, *Environmental Science and Pollution Research,* 5, 105-111, **1998.**
[36] Kiss, T., Odani, A., Demonstration of the importance of metal ion speciation in bioactive systems, *Bulletin of the Chemical Society of Japan,* 80, 1691-1702, **2007.**
[37] Laires, M. J., Monteiro, C. P., Bicho, M., Role of cellular magnesium in health and human disease, *Frontiers in Bioscience,* 9, 76, **2004.**
[38] Lukaski, H. C., Nielsen, F. H., Dietary magnesium depletion affects metabolic responses during submaximal exercise in postmenopausal women, *The Journal of Nutrition,* 132, 930-935, **2002.**
[39] Nelson, A. E., Chaudhary, S., Kraus, V. B., Fang, F., Chen, J. C., Schwartz, T. A., Shi, X. A., Renner, J. B., Stabler, T. V., Helmick, C. G., Caldwell, K., Whole blood lead levels are associated with biomarkers of joint tissue metabolism in African American and white men and women: The Johnston County Osteoarthritis Project, *Environmental Research,* 111, 1208-1214, **2011.**
[40] Nelson, A. E., Shi, X. A., Schwartz, T. A., Chen, J. C., Renner, J. B., Caldwell, K. L., Helmick, C. G., Jordan, J. M., Whole blood lead levels are associated with radiographic and symptomatic knee osteoarthritis: a cross-sectional analysis in the Johnston County Osteoarthritis Project, *Arthritis Research & Therapy,* 13, 1. **2011.**
[41] Taneja, S. K., Mandal, R., Assessment of mineral status (Zn, Cu, Mg and Mn) in rheumatoid arthritis patients in Chandigarh, India, *Rheumatology Reports,* 1, 5, **2009.**
[42] Goyer, R. A., Toxic and essential metal interactions, *Annual Review of Nutrition,* 17, 37-50, **1997.**
[43] Qayyum, M. A., Shah, M. H., Comparative assessment of trace metals in the blood of ovary cancer patients and controls, *Trace Elements & Electrolytes,* 32, **2015.**
[44] Rivai, I., Heavy metals in human hair related to age groups and automotive pollution levels of Bandarlampung city, Indonesia, *Bulletin of Environmental Contamination and Toxicology,* 66, 443-448, **2001.**
[45] Witte, K. K., Clark, A. L., Cleland, J. G., Chronic heart failure and micronutrients, *Journal of the American College of Cardiology,* 37, 1765-1774, **2001.**
[46] Yazar, M., Sarban, S., Kocyigit, A., Isikan, U. E., Synovial fluid and plasma selenium, copper, zinc, and iron concentrations in patients with rheumatoid arthritis and osteoarthritis, *Biological Trace Element Research,* 106, 123-132, **2005.**
[47] Burguera, J., Burguera, M., Anez, N., Changes in the total content of iron, copper, and zinc in serum, heart, liver, spleen, and skeletal muscle tissues of rats infected with Trypanosoma cruzi, *Biological Trace Element Research,* 37, 51-70, **1993.**
[48] Begovich, A. B., Carlton, V. E., Honigberg, L. A., Schrodi, S. J., Chokkalingam, A. P., Alexander, H. C., Ardlie, K. G., Huang, Q., Smith, A. M., Spoerke, J. M., Conn, M. T., A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis, *The American Journal of Human Genetics,* 75, 330-337. **2004.**
[49] Morgenstern, H., Machtey, I., Serum zinc and copper levels in rheumatoid arthritis, *Arthritis & Rheumatism,* 26, 933-934, **1983.**
[50] Naveh, Y., Schapira, D., Ravel, Y., Geller, E., Scharf, Y., Zinc metabolism in rheumatoid arthritis: plasma and urinary zinc and relationship to disease activity, *The Journal of Rheumatology,* 24, 643-646, **1997.**
[51] Dore-Duffy, P., Peterson, M., Catalanotto, F., Marlow, S., Ho, S. Y., Ostrom, M., Weinstein, A., Zinc profiles in rheumatoid arthritis, *Clinical and Experimental Rheumatology,* 8, 541. **1990.**
[52] Muller, H., de Toledo, F. W., Resch, K. L., Fasting followed by vegetarian diet in patients with rheumatoid arthritis: a systematic review, *Scandinavian Journal of Rheumatology,* 30, 1-10, **2001.**
[53] Pattison, D. J., Symmons, D. P., Lunt, M., Welch, A., Luben, R., Bingham, S. A., Khaw, K. T., Day, N. E., Silman, A. J., Dietary risk factors for the development of inflammatory polyarthritis: evidence for a role of high level of red meat consumption, *Arthritis & Rheumatism,* 50, 3804-3812, **2004.**
[54] Benito-Garcia, E., Feskanich, D., Hu, F. B., Mandl, L. A., Karlson, E. W., Protein, iron, and meat consumption and risk for rheumatoid arthritis: a prospective cohort study, *Arthritis Research & Therapy,* 9, 1, **2007.**
[55] Criswell, L. A., Merlino, L. A., Cerhan, J. R., Mikuls, T. R., Mudano, A. S., Burma, M., Folsom, A. R., Saag, K. G., Cigarette smoking and the risk of rheumatoid arthritis among postmenopausal women: Results from the Iowa Women's Health Study, *The American Journal of Medicine,* 112, 465-471, **2002.**
[56] Karlson, E. W., Lee, I., Cook, N. R., Manson, J. E., Buring, J. E., Hennekens, C. H., A retrospective cohort study of cigarette smoking and risk of rheumatoid arthritis in female health professionals, *Arthritis & Rheumatism,* 42, 910-917, **1999.**
[57] Krishnan, E., Sokka, T., Hannonen, P., Smoking-gender interaction and risk for rheumatoid arthritis, *Arthritis Research & Therapy,* 5, 1, **2003.**
[58] Padyukov, L., Silva, C., Stolt, P., Alfredsson, L., Klareskog, L., A gene-environment interaction between smoking and shared epitope genes in HLA-DR provides a high risk of seropositive rheumatoid arthritis, *Arthritis & Rheumatism,* 50, 3085-3092, **2004.**
[59] Raychaudhuri, S., Remmers, E. F., Lee, A. T., Hackett, R., Guiducci, C., Burtt, N. P., Gianniny, L., Korman, B. D., Padyukov, L., Kurreeman, F. A., Chang, M., Common variants at CD40 and other loci confer risk of rheumatoid arthritis, *Nature Genetics,* 40, 1216-1223, **2008.**
[60] Stolt, P., Bengtsson, C., Nordmark, B., Lindblad, S., Lundberg, I., Klareskog, L., Alfredsson, L., Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases, *Annals of the Rheumatic Diseases,* 62, 835-841. **2003.**

[61] Sverdrup, B., Källberg, H., Bengtsson, C., Lundberg, I., Padyukov, L., Alfredsson, L., Klareskog, L., Association between occupational exposure to mineral oil and rheumatoid arthritis: results from the Swedish EIRA case–control study, *Arthritis Research & Therapy,* 7, 1, **2005.**
[62] Costenbader, K. H., Feskanich, D., Mandl, L. A., Karlson, E. W., Smoking intensity, duration, and cessation, and the risk of rheumatoid arthritis in women, *The American Journal of Medicine,* 119, 503e1, **2006.**
[63] Chatterjee, M., Saluja, R., Kanneganti, S., Chinta, S., Dikshit, M., Biochemical and molecular evaluation of neutrophil NOS in spontaneously hypertensive rats, *Cellular and Molecular Biology*, 53, 84-93, **2006.**
[64] Shrivastava, R., Upreti, R. K., Seth, P. K., Chaturvedi, U. C., Effects of chromium on the immune system, *Immunology & Medical Microbiology,* 34, 1-7, **2002.**
[65] Ozgunes, H., Gurer, H., Tuncer, S., Correlation between plasma malondialdehyde and ceruloplasmin activity values in rheumatoid arthritis, *Clinical Biochemistry,* 28, 193-194, **1995.**

6. ACKNOWLEDGEMENTS

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